Investigating tumour-mediated immunosuppression of CD8+ T cells.

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MSc(by research)

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Author's Declaration

"This project is my own work except where indicated. All text, figures, tables, data or results, which are not my own work, are indicated and the sources acknowledged. In addition, I confirm that the hardcopy and the e-submission are identical."

Signed: Daniel Novelli Date:17/07/2019

Abstract

Prostaglandin E2 is a physiologically active lipid mediator. However, an upregulation of PGE_2 is often seen within the tumour microenvironment of multiple cancer types, helping drive multiple aspects of tumour development. PGE_2 has a versatile role in aiding tumour development and progression, including evasion of the immune system. Cytotoxic T cells play a key role in eliminating cancer cells, preventing tumour growth and development, through the specific recognition of tumour specific antigens and subsequent cytolytic function. Studies have shown 1uM PGE_2 to suppress both the proliferation and effector function of naive cytotoxic T cells, preventing their cytotoxic function, in turn enabling cancer development. We aimed to determine the full extent of PGE_2 induced immunosuppression of cytotoxic T cells(CTLs) in addition to the potential causative mechanism. Live cell microscopy imaging determined whether PGE_2 affected the stability and maintenance of the immunological synapse, as a recently identified potential common mechanism of immune suppression. Flow cytometry analysis was used to investigate the effect of PGE_2 on both proliferation and cytolytic action of CTLs. In addition, imaging-based kill assays were used to further confirm the effect of 1uM PGE_2 on CTL cytolysis against Renca mCherry cells. Results showed PGE_2 to have no significant effect on the stability of the immunological synapse between CTL and Renca targets. In contrast 1uM PGE_2 was shown to induce significant decreases in both the proliferation and cytotoxicity of CD8+ T cells, in a non-dose dependant manner. Partial restoration of suppression was noted following the treatment of EP2 blocker TG4-155. However, the suppressive effect was completely abrogated through the addition of supplementary IL-2 into T cell culture. Overall, PGE_2 was concluded to have a significant immunosuppressive effect against CTLs and therefore could potentially contribute to tumour mediated immunosuppression.

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Abbreviations

AA	Arachidonic acid
AC	Adenyl Cyclase
AIRE	Autoimmune regulator transcription factor
APC	Antigen presenting cell
ATP	Adenosine triphosphate
EBV	Epstein Barr Virus
cAMP	Cyclic adenosine monophosphate
CFSE	Carboxyfluorescein succinimidyl ester
COX	Cyclooxygenase
CTLA-4	Cytotoxic T lymphocyte associated protein 4
CTV	Cell trace violet
DAG	Diacylglycerol
DC	Dendritic cell
DN	Double negative
DP	Double positive
FMO	Fluorescence minus one
FoxP3	Forkhead box P3
HA	Haemagglutinin
Hep-B	Hepatitis B
HPV	Human Papilloma Virus
IFN	Interferon
IL	Interleukin
IP3	Inositol triphosphate
LAG-3	Lymphocyte activation gene-3

LB	Luria-Bertani
MACS	Magnetic activated cell sorting
MFI	Median intensity fluorescence
MHC	Major histocompatibility complex
NFAT	Nuclear factor of activated T cells
NK	Natural killer cells
PD-1	Programmed death receptor 1
PFA	Paraformaldehyde
PGE_2	Prostaglandin E2
PLC	Phospholipase C
RAG	Recombination activating genes
Renca	Murine renal carcinoma
SMAC	Supramolecular activation cluster
SP	Single Positive
TAA	Tumour associated antigen
TCR	T cell receptor
TIGIT	T cell immunoglobulin and ITIM domain
TIL	Tumour infiltrating lymphocyte
TME	Tumour microenvironment
TNF	Tumour necrosis factor
Treg	Regulatory T cell
WT	Wild type

Antibodies Used

Antibody	Conjugated fluorochrome:	Target	Clone	Dilution	Supplier
MHC1(H2Kd)	Pacific Blue	Extracellular stain for the detection of H2Kd(flow cytometry)	SF1-1.1	1:200	Biolegend
MHC2	APC	Extracellular stain for the detection of MHC2(flow cytometry)	M5/114.15.2	1:400	Biolegend
CD40	PE	Extracellular stain for the detection of CD40(flow cytometry)	3/23	1:20	Biolegend
CD80	BV650	Extracellular stain for the detection of CD80(flow cytometry)	16-10A1	1:40	Biolegend
CD86	BV605	Extracellular stain for the detection of CD86(flow cytometry)	GL-1	1:40	Biolegend
ICAM-1	FITC	Extracellular stain for the detection of ICAM-1(flow cytometry)	3E2	1:200	BD Bioscience
Anti-mouse CD279(PD-1)(Clone 29F.1A12)	BV786	Extracellular stain for the detection of PD-1(flow cytometry)	M1H5	1:200	Biolegend
Anti-mouse CD274(PD-L1)(Clone MIH5)	BV421	Extracellular stain for the detection of PD-L1(flow cytometry)	M1H5	1:200	BD Bioscience
Galectin-9	BV421	Extracellular stain for the detection of Galectin-9(flow cytometry)	RG9-35	1:100	BD Bioscience
Thy1.1	PerCP	Extracellular stain for the detection of Thy1.1(flow cytometry) – Identification of lymphocytes	OX-7	1:100	BD Bioscience
CD8a	APC	Extracellular stain for the detection of CD8b(flow cytometry) – Identification of CD8+ T cells	53-6.7	1:100	Biolegend
Vb8.1	FITC	Extracellular stain for the detection of CD8b(flow cytometry) – Identification of CL4 CD8+ T cells	KJ16-133	1:100	Invitrogen
Zombie NIR	APC-Cy-7	Live / Dead stain	-	1:100	ThermoFisher Scientific
PI	PI/PE	Live / Dead stain	-	1:400	ThermoFisher Scientific
Cell Trace Violet	445_440	Target cell identification/proliferation assay	-	1:1000	ThermoFisher Scientific
Lck 418	AF488	Intracellular stain for phosphorylated Lck 418 – indicative of phosphorylated Lck 394	K48-37	1:100	BD Bioscience
Lck 505	AF647	Intracellular stain for phosphorylated Lck 505	4/LCK-Y505	1:100	BD Bioscience
Lck Total	PE	Intracellular stain for phosphorylated Total Lck phosporylation	MOL171	1:100	BD Bioscience

1. Introduction

1.1 Cancer

1.1.1 Overview

Cancer is a term used to describe a group of over 200 individual diseases, characterised by uncontrolled and genetically unstable proliferation of cells, which have the potential to invade tissues and spread throughout the body.

Every year an additional 360,000 people are diagnosed with cancer in the UK alone(Cancer Research UK, 2012). With an increasing incidence, due to a combination of the ageing population and environmental factors, cancer will affect 1 in 2 people globally, at some point throughout their life time. Insight of this, cancer research has grown dramatically over the past 4 decades, with survival rates doubling to 50%(Cancer Research UK, 2012). The increased survival rate is primarily due to a vast improvement in diagnostics, the identification of prognostic markers and overall treatment methods. However as of 2016, cancer reached an annual death rate of 164,782 in the UK alone(Macmillan Caner). Cancer still proves to be one of the biggest killers in the world, accounting for 28% of deaths annually in the UK(Cancer Research UK, 2012).

Mitosis of eukaryotic cells is a vital process and is therefore controlled by many meticulous checkpoints to ensure that the DNA is replicated accurately to sustain equal genetic DNA in both daughter cells. However, a gradual accumulation of specific mutations can lead to the loss of one or more of these checkpoints and DNA damage repair mechanisms, otherwise known as tumour suppressor gene. Mutations also lead to the activation of sustained proliferation mechanisms, known as proto oncogenes(1)

Due to the novel genetic instability of the cancer cell genome, further mutations accumulate giving these transformed cells a survival or growth advantage. These additional mutations can give

rise to invasion and spread of cells throughout the body, in a process named metastasis. These cells would be classified as cancerous and possess a group of phenotypic properties or "hallmarks" that enable their development and progression, as seen in Figure 1(2).

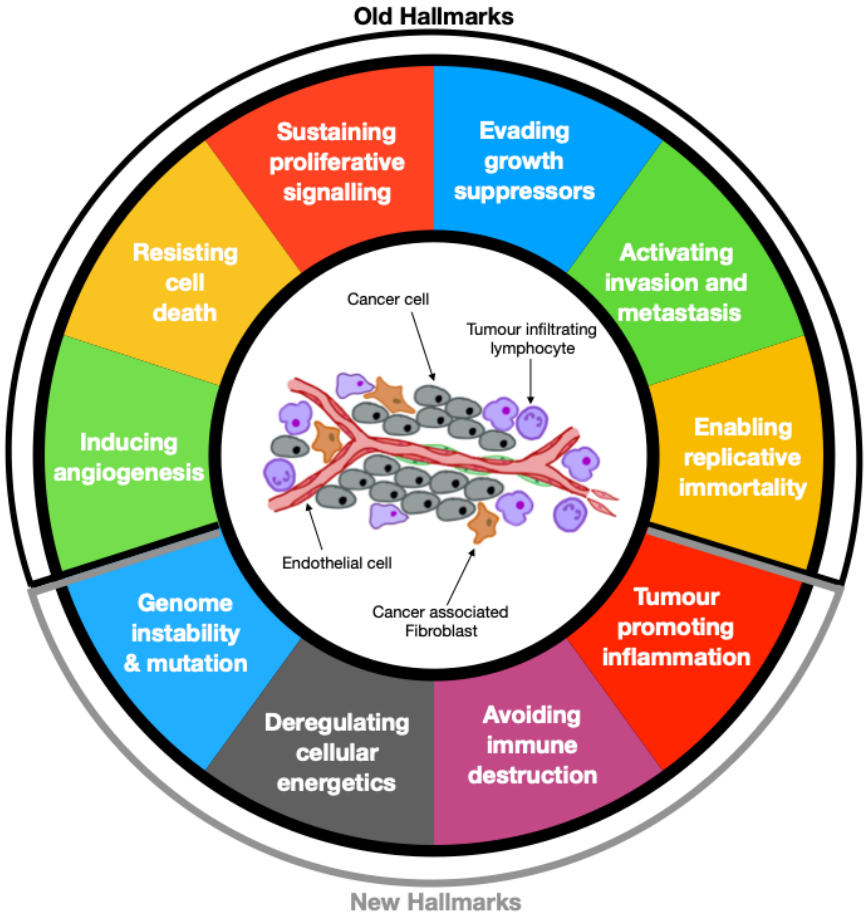


Figure 1 : Hallmarks of cancer
The select group of phenotypic attributes that allow the formation of a tumour. Each hallmark holds a vital role in tumour formation, therefore producing a potential set of therapeutic targets to overcome. *Adapted from "The Hallmarks of Cancer: the next generation" - Hanahan D, Weinberg RA.*

1.1.2 Hallmarks of cancer

Malignancies develop due to the progressive evolution of multiple mutations leading to an unstable genome and the acquisition of a group of specific capabilities that enable the tumour pathogenesis. These attributes include:

- **Resistance to cell death:** Cancer cell development must overcome many of the cellular mechanisms leading to apoptosis. In contrast to necrosis, apoptosis is an organised, ubiquitous

cellular suicide mechanism. Apoptosis can be induced in response to both the internal and external stimuli via intrinsic or extrinsic pathways, respectively(3). Environmental stresses such as DNA damage, hypoxia and signalling imbalances typically lead to induction of apoptosis to prevent loss of genetic integrity. However, cancer cells are under constant oncogenic stress, due to cell cycle arrest mechanisms attempting to contradict activated oncogene action, and therefore must develop mechanisms to prevent apoptosis, in particular the intrinsic pathway(4). Apoptosis is ultimately a multi-caspase driven mechanism, intrinsically mediated through the imbalance of apoptotic signalling molecules. Increased levels of pro-apoptotic protein signalling molecules, e.g. Bak and Bax, targeting the mitochondrial membrane induces apoptosis. Once bound to the membrane Bax molecules cluster inducing a pore that enables the release of cytochrome into the cytoplasm(5). In contrast, cancer cells common up regulate anti-apoptotic signalling molecules, such as BCL-2, to tip the balance in favour preventing apoptosis via competitive inhibition(6).

- **Induction of angiogenesis:** Solid tumour formation leads to the production of an 3D mass of cells. Local circulation is sufficient to provide nutrients to a tumour up to 2mm in diameter however anything larger results in an outer shell of cells encasing a hypoxic core as the vital factors, such as oxygen, cannot reach the inner cells through simple diffusion(7). One mechanism a tumour overcomes the lack of sufficient oxygen is the development of novel blood vessels, through a process referred to as angiogenesis(8). Angiogenesis can lead to altered genetic profiles of the inner malignant cells. The up regulation of Hypoxia induced factor-1(HIF-1) expression is seen within cells facing hypoxic conditions(9). Increased levels of this gene promoter can lead to increased expression of Vascular endothelial growth factor(VEGF), resulting in angiogenesis throughout the tumour, enabling further growth(10).

- **Replication immortality:** Cells within the body undergo varied levels of mitosis depending on their origin, cell type and purpose. However most eukaryotic cells have a limited number of

mitotic divisions, the exception being stem cells. Mitotic divisions are tightly controlled through multiple mechanisms including the DNA damage response and shortening of telomeres(11). Mutations in cellular mitotic checkpoint controls allows for the unlimited capability of cellular proliferation, in turn leading to tumour formation. Most eukaryotic cells reach a stage of senesce following a limited number of divisions. This is achieved through multiple intrinsic triggers. One mechanism used is the shortening of telomeres. Telomeres are repeated base pair sequences found at the end of chromosomes to protect the DNA. Each cellular division further shortens the cell's telomeres as the initiating replication primer does not recognise the end of the telomere(12). Once the telomere reaches a critical length the DNA damage response recognises the telomere as exposed DNA damage triggering senescence. Many cancers overcome this through the modification and up regulation of telomerase enzymes that allow extension of the preexisting telomeres, preventing senescence(13). More commonly, mutations occur in the genes controlling the DNA damage response. P53, a key tumour suppressor gene, is named the guardian of the genome and is the most commonly suppressed protein due to mutation seen in cancer(14). P53 is activated in response to DNA damage, halting the cell cycle until the damage is resolved otherwise inducing apoptosis. The role of P53 in halting the cell cycle is completed to ensure correct and accurate DNA replication is completed, maintaining genetic integrity. "Loss of function" base pair mutations that lead to the partial or complete loss of protein function, are commonly seen in TP53, leading to the continuation of cellular proliferation despite DNA damage, allowing multiple mutations to occur due to the natural DNA polymerase error rate, as well as bypassing the apoptotic effect of critical length telomeres; driving further carcinogenesis(15).

- **Evasion of growth signals:** Growth signals such as cytokines, chemokines and prostaglandins underpin the molecular signalling mechanisms of the cells within the body. They enable detection and transduction of external conditions within the cellular microenvironment, informing cells when to alter their metabolic rate, undergo mitosis and when to remain quiescent. Cancer cells

gain the ability to evade the necessity of these mediators allowing their growth and proliferation in all environments(16). One mechanism by which cancer cells achieve this is through genetic mutations causing the increased expression of growth factor receptors, such as the tyrosine kinase EGFR(17). By increasing the expression of growth factor receptors, the cells can activate pro-replicative signalling cascades in even the lowest concentrations of growth factors(18). In addition, many neoplastic cells down regulate receptors of suppressive markers, inhibiting suppression of their cell growth(19).

- **Invasion and metastasis:** A defining characteristic of cancer is its ability to spread throughout the body via two primary mechanisms: invasion and metastasis. Invasion defines the ability of the proliferating malignant cells to penetrate into neighbouring tissues(20). For invasion to occur the cancer cells must first overcome tight cell to cell interactions, predominantly 'Tight Junctions'. Located on the apical membrane, tight junctions enforce adjacent cellular contacts that occlude the intercellular space, in turn, creating a barrier and preventing cell migration. This is completed through multiple protein interactions such as occludins. Studies have revealed that there is a significant down regulation of these proteins in cancer. One supporting study of 124 cancerous and 33 normal breast tissues found a significant down regulation of occludin proteins in metastatic disease(21). Once overcoming cellular adhesions, the transformed cells are seen to secrete paracrine enzymes that degrade basement membrane and extracellular matrix, enabling local spread of the tumour encompassing adjacent tissues and organs, named invasion. Metastasis is the ability of a cancer cell following invasion to enter circulation and migrate to a distant site initiating a secondary or even tertiary tumours(22). It occurs through a series of events, called the metastatic cascade.

More recently it has been suggested that 4 additional 'hallmarks' substantially contribute towards development and pathogenesis of cancer. The 4 novel hallmarks consist of: Genome instability and

mutation, Deregulation of cellular metabolism, Tumour promoting inflammation and almost paradoxically evasion of the immune system(23). Evasion of the immune system, especially the directly cytotoxic cells such as NK cells and CD8+ T Cells, is essential in both primary tumour formation and metastasis(24). Cancer cells are typically highly immunogenic due to the multitude of mutations causing the expression and presentation of altered or even completely new proteins. The expression of adapted proteins allows for cancer cell recognition and elimination by the immune system, in a process called immunosurveillance(25). However, the immune elimination of cancer cells also acts as a selection pressure, driving further evolution of the transformed cells creating mechanisms that bypass recognition and elimination by the immune system. Immuno-edited malignant cells are then able to proliferate forming a tumour and ultimately cancer.

1.2 The Immune system

1.2.1 Introduction

The immune system is a complex system comprised of multiple cell types, cooperatively functioning to protect the body against harmful pathogens as well as malignant cells(26). The advanced diversity of the immune system is enabled through multiple developmental rearrangement mechanisms that produce a wide variety of protein receptors combinations, capable of recognising and activating cytotoxic functions against foreign peptide fragments named antigens. In addition, the immune system can mount a memory response to previously detected antigens. Immune memory enforces a heightened, quicker and therefore more efficient response against re-occurring infections, known as immunity(27). The system includes multiple aspects including both a nonspecific and specific response, named Innate and Adaptive respectively.

1.2.2 Innate

The innate immune response is the body's nonspecific defence against antigens. This branch of immunity is not specific to any particular antigen but instead is an initial defense upon exposure to pathogens, allowing for a quick and broad prevention of infection. The innate system is comprised firstly of physical barriers, such as the skin, and chemical mediators, such as mucus and acid(28). The innate immune system is also composed of a cellular component, namely leukocytes. Leukocytes, also known as white blood cells, includes both phagocytic antigen-presenting cells e.g. Dendritic cells and macrophages, as well as cytotoxic cells e.g. NK cells. Recognition of foreign antigens, such as LPS, along with activating double molecules enforces a strong innate response against immunogenic pathogens.

1.2.3 Adaptive

The adaptive immune response is a specific, targeted response produced post-exposure to antigen. Consisting of two main branches, cellular and humoral, the adaptive immune system plays a role in detecting and eliminating, whilst remembering, pathogens that have breached outer epithelium. The adaptive immune system can be activated in multiple ways including antigen presentation by innate professional antigen presenting cells(APCs). APCs endocytose pathogens into their cytoplasm before degrading them into short peptides using the proteasome. Once degraded, 8-16 amino acid long peptides are then transported into the lumen of the endoplasmic reticulum via the TAP protein complex(29). Peptides that successfully bind to MHC1 or MHC2 complexes then leave the ER and are presented on the cell surface for interaction with naive lymphocytes in the local draining lymph node.

There are two classes of lymphocytes: B cells and T cells. B cells are both produced and developed within the bone marrow before migrating to the lymphatic system, either the spleen or

lymph nodes, where they reside until they are activated. Upon activation B cells can undergo two primary immune response. B cells can detect foreign antigens via specific membrane bound immunoglobulins, deemed B cell receptors. When exposed to an antigen a select group of B cells migrate out of the lymph nodes, into the circulation where they differentiate into short lived plasmablasts, enabling them to secrete antibodies to aid immune recognition, neutralisation of toxins and cytotoxicity towards pathogens(30). Alternatively, B cells reside in the lymph nodes forming germinal centres, where they are able to undergo both class switch recombination and somatic hypermutation to drive the immense diversity of antibody recognition.

T cells are produced in the bone marrow but develop in the thymus before entering circulation. During development all T Cells produce a unique T cell receptor(TCR) consisting of 2 polypeptide chains, most commonly an alpha and beta chain(31). T Cells use their TCR to detect specific antigens presented by either a Class 1 or Class 2 Major Histocompatility Complex(MHC1) from the target cells. There are a variety of T cells, each playing a vital role in protecting the body. The three main classes being: T helper cells, T regulatory cells and Cytotoxic T cells. T helper cells, also known as CD4+ T Cells, express the CD4 co-receptor to aid target cell interaction. CD4+ T cells are primarily involved in influencing the action of other immune cells, in either a positive or suppressive manner, through the release of cytokines(32). On the other hand, T regulatory cells predominant role is to down regulate immune responses, such as CD8+ T cells, and maintaining immune tolerance(33). Lastly CD8+ T cells are essential in the prevention of intracellular infection and tumour formation.

1.3 Cancer Immunosurveillance

1.3.1 Cancer Immunosurveillance

The first description of the anti-tumour effects of the immune system was provided by William Coley in 1891, when he demonstrated that injecting attenuated pathogenic bacteria, *Streptococcus*

pyogenes and *Serratia marcescens,* into a tumour lead to regression(34). This was thought to be due to a bystander effect of stimulation of the immune system. However due to the high risk associated with using pathogenic microorganisms as a treatment, the development of surgery as well as chemo and radiotherapy surpassed this hypothesis leaving little progression of the treatment, until the mid-20th century. Thomas and Burnet first described the immuno-surveillance theory in 1957(35). Due to the multifaceted process of cancer development, the immune system holds many essential roles in the prevention of tumour formation.

One way in which the immune system prevents cancer development is the quick and efficient elimination of pathogens, preventing of inflammation and damage of host cells. Inflammation is a key process in the body's recovery process following injury and infection however it has also been demonstrated to significantly contribute to tumour development. Chronic inflammation has been shown to increase the risk of tumour development(36). Inflammation can help drive carcinogenesis by inducing breaks in the DNA of transformed cells leading to an increased mutation rate(37). Additionally, it has been suggested that inflammation can aid tumour metastasis by degrading basement membranes and extracellular matrixes, allowing for intra/extravasion of metastatic cells(38). Therefore, the efficient regulation of inflammation following infection is essential in cancer prevention.

An additional process the immune system completes that prevents cancer development is the detection and elimination of viral infections. Viruses can contribute to the development of cancer in two main ways. The primary way in which viruses can induce and progress cancer development occurs through the mechanism of infection and inflammation previously described, such and HEP-B(39). In addition to this, many viruses have been identified to directly cause cancer development such as HPV and EBV(40). This occurs through the insertion of oncogene encoding viral DNA into the host cells, resembling many of the naturally mutated oncoproteins seen in non-viral cancer(41). In both cases it is vital that the immune system can detect and eliminate all viruses that infect host cells in order to prevent the development of tumours.

Lastly is the detection of naturally transformed cells. Mutations do occur within the cells of our body following proliferation, due to naturally occurring errors in replication polymerases(42). It is speculated that on average eukaryotic DNA polymerases create an error in 1 in every 10^5 bases, however the majority of these are corrected through the DNA damage response, ultimately leading to a spontaneous mutation rate of approximately 10^{10} per base pairs per replication cycle(43). Most mutations are missense and shall have little to no effect on our genome. However, transforming mutations can occur, leading to transformed and potentially cancerous cells. It is important that the immune system can detect any transformed cells in order to eliminate them before they have the opportunity to proliferate forming a tumour. This process is called cancer immunosurveillance and involves both the innate and adaptive branches of the immune response(44). There are 3 primary phases of immunosurveillance, as seen in figure 2.

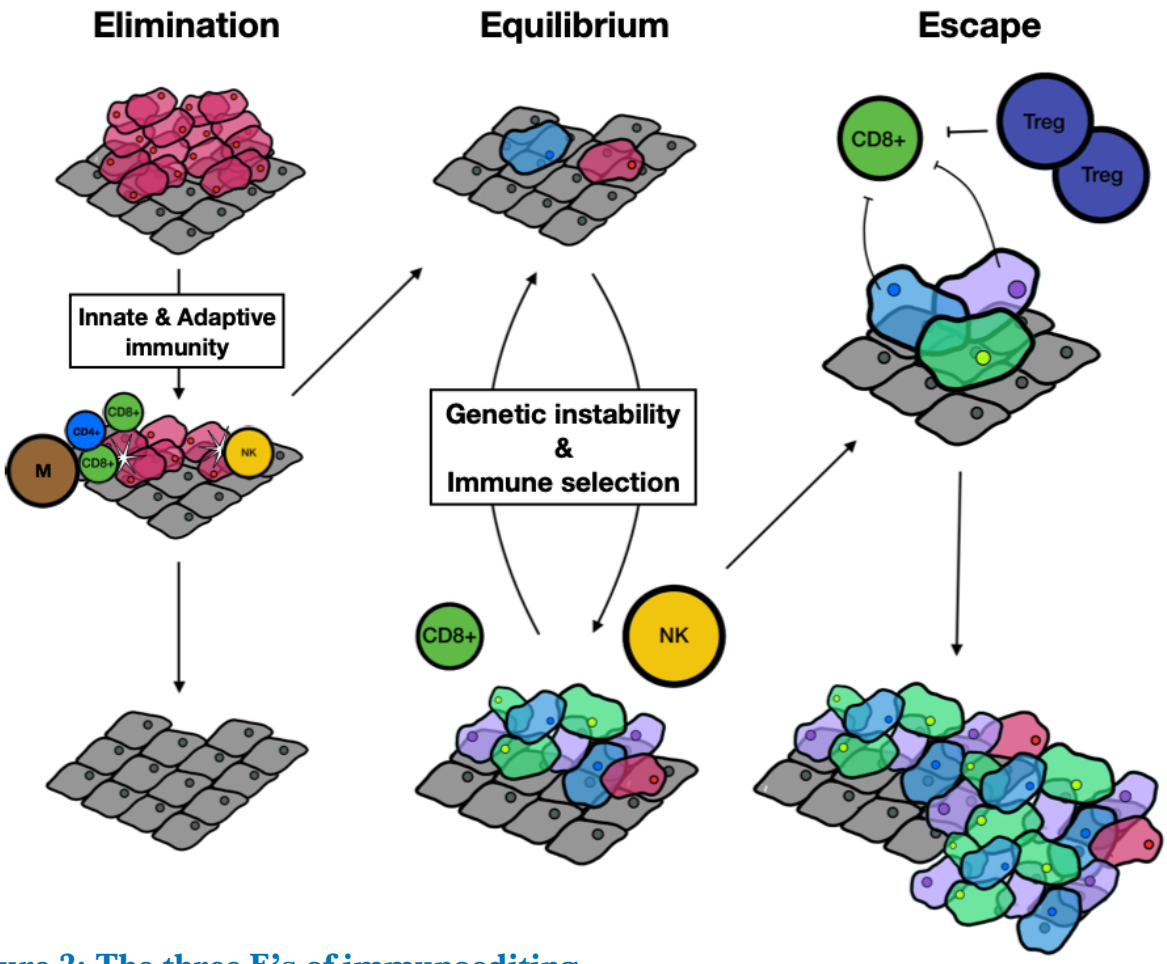


Figure 2: The three E's of immunoediting

The three alternative relationships noted between cancer cells and the immune system. Primarily the immune system is able to detect and eliminate naturally transformed cells preventing tumour development. Cancer cells develop mechanisms to evade immune detection, however the main bulk of the tumour is still eliminated, leaving individual transformed cells, resulting in an equilibrium phase. An accumulation of additional mutations enables cancer cells to develop suppressive mechanisms, in turn, further evading the immune system until complete escape is complete and a tumour forms. *Adapted from Strausberg, Genome Biol. (2005).*

1.3.2 Elimination

In the primary phase of immunosurveillance, transformed cells have not yet acquired sufficient mutations to outgrow the rate of elimination by the immune system. Many of the mutations that arise during or as part of the process of carcinogenesis also lead to the production of altered/novel proteins making the cells highly immunological. Alternatively, some cancer cells down regulate specific MHC complexes, making them susceptible to NK cell cytolysis(45). Cytosolic novel and altered proteins are degraded by the proteasome into short peptides approximately 8-16 amino acids long. These peptides are transported into the Endoplasmic Reticulum(ER) via the TAP protein(46). Once in the ER the degraded peptides associate with an MHC1 complex. Is successfully associated the MHC1 and antigen complex are expressed on the cells surface. These peptides can be directly recognised by cytotoxic T cells in situ or indirectly recognised when presented by APCs, such as mDC cells, in the tumour draining lymph node, through cross presentation(47). Recognition by CTLs leads to elimination of the transformed cells.

1.3.3 Equilibrium

Equilibrium is an intermediate phase, which is accomplished when the genetic instability of the transformed tumour cells has lead to the development mechanisms enabling increased replication rates and/or a loss of immunogenicity, by down regulation of cell surface markers, preventing complete detection and elimination by the immune system(48). However, the cells have not developed enough evasive mechanisms to completely escape the immune system leading to a system in which the tumour cannot increase in size as it is being killed at the same rate it is proliferating, leaving a relatively low number of surviving transformed cells. This process shall continue to occur in a dynamic equilibrium, with the immune system acting as a selection pressure, until the transformed cells acquire additional mutations to further prevent elimination by the immune system, which in turn will lead to the "Escape" phase of immunoediting/evolution.

1.3.4 Escape

Despite immunosurveillance mechanisms tumours are still able to develop due to an accumulation of further mutations reducing the immunogenicity of the cancerous cells and causing tumour mediated immune suppression. Cancer immune evasion is contributed to through many mechanisms such as decreasing their immunogenicity, antigenic modulation and tumour-induced immune suppression. For a cytotoxic T cell to kill a target cell it must first be able to recognise the class 1 major histocompatibility complex-bound antigen being presented, become stimulated through co-stimulation, and form a stable immune synapse through co-signalling molecules. Tumour cells exploit this mechanism by altering their cell surface expression to deter recognition and therefore elimination via immune cells(49). This has been shown to be done using three main strategies.

One mechanism cancer cells use to evade the immune system is the down regulation of MHC complexes. MHC1 expression is essential for the recognition of antigens and therefore cytolytic function of CD8 T cells. By down regulating the expression of MHC1, tumour cells evade recognition by the adaptive immune system preventing their death(50). However, NK cells exhibit a unique ability to be able to detect cells that express low or even undetectable levels of MHC1 complexes(51). The mechanism behind NK recognition is still not fully understood however it is believed that NK cells express an inhibitory receptor that recognises MHC1 alleles, enabling their cytotoxic ability only when faced with a cell expressing low levels of MHC1.

Another mechanism of immune evasion by cancer cells is the loss of expression of co-signalling molecules, primarily CD80 and CD86 (52). Both these molecules are typically required for both the initial priming and activation of cytotoxic T cells. By losing CD80/CD86 expression the tumour is not only able to inhibit activation and therefore cytolytic action, but also induce anergy in CD8+ T cells. However, T cells can utilise adhesion molecules, specifically ICAM-1 and LFA-3, as a co-stimulatory signal to undergo priming and activation. CTLs express LFA-1, a cell adhesion molecule historically known to induce emigration of lymphocytes across blood vessel walls and into

tissues. More recently LFA-1 has also been associated with the activation and priming of CD8 T cells when bound to ICAM-1. However, the loss of adhesion molecules, such as ICAM-1, in cancer cells has been noted decreasing migration of CTLs into the tumour as well as preventing activation.

An additional mechanism used my tumour cells to evade CTL or NK cell lysis is through endocytosis of molecules that many increase the cell surface immunogenicity(53). This is especially important in preventing antibody-dependant cellular cytotoxicity and complement. Both immunity mechanisms utilise the humoral aspects of the immune system to highlight target cells, making them more easily recognisable by cytotoxic cells of the immune system. To deter this, the tumour cells have been shown to endocytose and degrade the attached antibodies inhibiting them from activity cytotoxic cells.

Lastly, is the tumour ability to directly inhibit cytotoxic immune cells either by the up regulation of inhibitory cell surface proteins, such as PD-L1, or secretion of immune-suppressive factors, such as adenosine and PGE_2, later described.

1.4 CD8+ T cells

1.4.1 CD8+ T cells

Cytotoxic T Cells(CTL) express distinct T cell receptors that recognise a specific antigenic peptide bound to MHC1. Additionally, CTLs express a dimeric CD8 co-receptor comprised of CD8α and CD8β polypeptide chain(54). MHC class 1 molecules are expressed on the surface of all nucleated cells of the body as well as platelets(55). They are used to present fragments of degraded intracellular peptides. This enables the immune system to detect any compromises in the internal cellular environment, such as viral infection or malignancy. CD8+ T cells have the intrinsic ability to directly induce cell death through multiple mechanisms upon recognition of a MHC1 bound

antigen and co-stimulation, making them essential in the prevention of cellular infection and tumour formation.

1.4.2 T cell development

T cell progenitors differentiate from haematopoetic stem cells, in the bone marrow before they localise, via the blood, to the cortex of the thymus. Once in the thymus they are classified as thymocytes. Thymocytes undergo a series of maturation steps to ensure the production of effective T cells, as seen in figure 3.

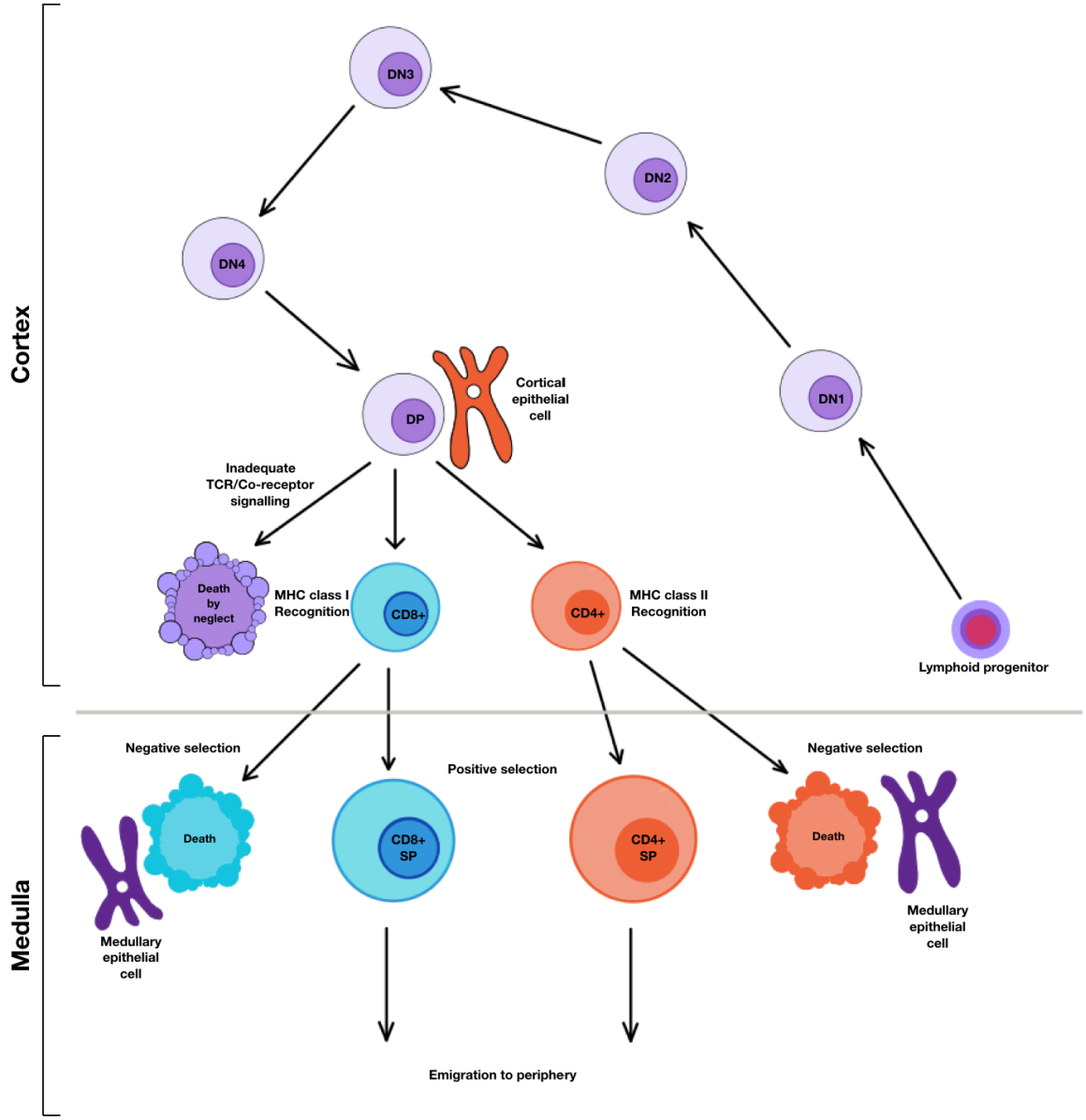


Figure 3: T cell development

The first step occurs in the cortex. At this stage the T cells lack both CD4 and CD8 co-stimulatory receptors and are therefore classified as "Double Negative"(56).

At this stage double-negative cells give rise to both γδ and αβ T cells(57). DN T cells must first generate a functioning TCR beta chain, through a process called VDJ recombination. VDJ recombination is the somatic genetic rearrangement within three distinct gene loci: the variable region, the diversity region, and the joining region(58). Rearrangement of these loci create the extensive diversity of TCR beta chains contributing to the specific recognition of an immense number of peptides. VDJ rearrangement is controlled by two Recombination Activating Gene enzymes, deemed RAG1 and RAG2(59).

Following VDJ rearrangement the resulting unique TCR beta chain is coupled with a surrogate TCR alpha chain forming the pre-TCR(60). Once formed the pre-TCR associates with a complex of peptides, named CD3. CD3 is a co-receptor made-up of four unique polypeptide chain, combined in 3 different ways, and is essential for proximal signal transduction(61). Formation of the TCR-CD3 complex allows for TCR signalling. If successful TCR signalling is transduced the T Cell is able to undergo proliferation, survival and further maturation. At this stage T cells with a non functioning TCR beta chain shall undergo apoptosis, ensuring only functioning TCR cells survive. The surviving cells undergo rearrangement of the TCR alpha chain via the same RAG driven VDJ mechanism, further increasing the diversity of specific TCRs.

Once a fully mature TCR has been established, additional genetic differentiation leads to the up regulation of both CD4 and CD8 co-receptors. The T Cells would now be considered "Double Positive". Surviving T cells with a fully functioning αβ-TCR undergo positive selection. Positive selection entails the exposure of the T cell to a MHC1 bound self antigen to ensure a fully functioning αβ TCR has been produced. Cortical epithelial cells present a small array of self peptides bound to both MHC1 and MHC2 complexes. For successful progression via survival signalling, TCRs must be able to adequately recognise these complexes. Studies have calculated that at this stage approximately 90% of DP T cells undergo death by neglect due to unsustained interaction with the peptide MHC complex(62).

Following successful recognition, lineage specific differentiation occurs causing the down regulation of either CD4+ or CD8+, generating a "Single Positive" T cell. T cells that solely express CD8+ co-receptor are deemed Cytotoxic T Cells(CTLs). Single positive T cells migrate from the cortex to the thymus medulla to undergo negative selection. This process is named central tolerance and is necessary to eliminate any TCRs with a high affinity to self-peptides, in order to prevent autoimmune disease. Medullary thymic epithelial cells present a multitude of self-peptides, produced by the enzyme AIRE, on both MHC1 and MHC2 complexes(63). Any CTLs with a strong affinity to the self-peptide complexes are eliminated via apoptosis. T cells that are capable of recognising the self peptide with a lower affinity progress, exiting the thymus and entering circulation. *Figure adapted from Germain, Ronald N. "T-cell development and the CD4–CD8 lineage decision." Nature Reviews Immunology 2 (2002): 309-322.*

1.4.3 CD8+ T cell activation

Stimulation of both the TCR and co-receptors results in a signalling cascade ultimately determining the fate of the T Cell, such as proliferation, survival, apoptosis or cytokine production. For

activation to be successfully completed CTLs must receive both primary and co-stimulation(64). First, is the recognition of an MHC1 presented antigen by the generated specific TCR. If successful, TCRs within the cell surface membrane undergo clustering, leading to the formation of a complex structure containing TCRs, co-signalling molecules, and adhesion molecules. This structure is named the supramolecular adhesion complex(SMAC)(65).

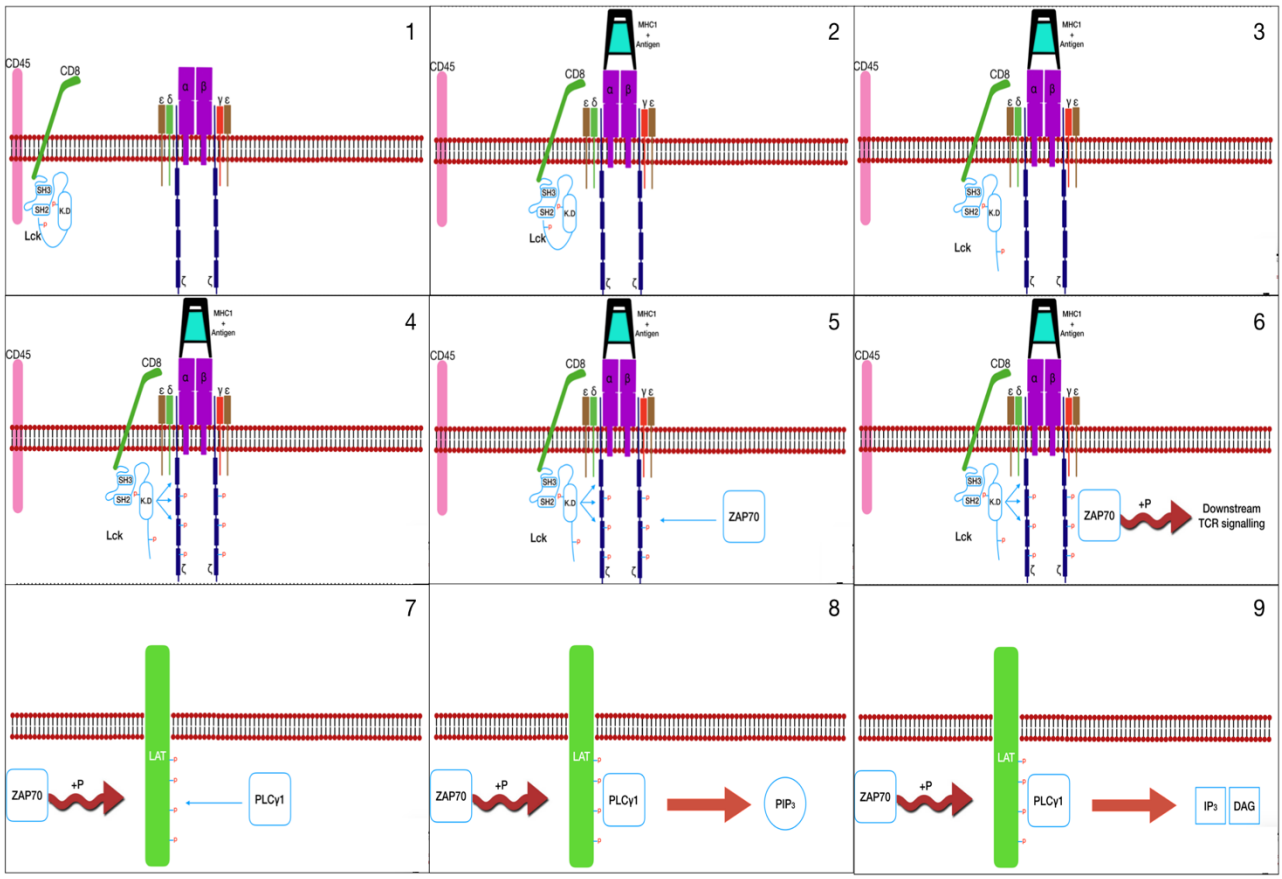


Figure 4 : T cell signalling

The process of redistribution is controlled by modifications in the actin cytoskeleton, and creates the foundation for an intracellular signalling cascade that ultimately determines the fate of the T cell(66). Clustering of CD8 and CD28 co-signalling molecules to the TCR recruits the membrane bound tyrosine kinase Lck, away from the protein tyrosine phosphatase - CD45(67-68). High concentrations of CD45 dephosphorylates the stimulatory phosphorylation on Tyrosine 394, leaving phosphorylated Tyr505 and therefore a constitutively inhibited Lck. In contrast low levels of CD45 are capable of dephosphorylation of Y505 leading to the activation of Lck. Clustering enables the migration and compartmentalization of Lck from CD45, reducing its levels in turn enabling activation of Lck by transphosphorylation.(69)

Recruitment of Lck to the SMAC, leads to the phosphorylation of the intracellular domain immuno-receptor tyrosine-based activation motifs(ITAMs) on CD3-ε chains. This phosphorylation attracts the recruitment of Zeta-chain associated protein kinase(ZAP-70) to both the CD3-ε and TCR-ζ, via Src homologous interaction(70). ZAP-70 is a 70kDa Syk family protein tyrosine kinase. ZAP-70 is able to activate the scaffold linker protein LAT, through phosphorylation of its multiple tyrosine residues. Activated LAT recruits and binds Slp76 via an adaptor protein, called Gads. Slp76, also known as lymphocyte cytosolic protein 2, is a vital signal transducing adaptor protein. This recruitment process is essential as it brings the Slp76 protein into close enough proximity to ZAP70 to be phosphorylated activating the LAT-Slp76 complex(71). This activated complex is responsible for recruiting and activating a variety of further signalling cascades, transducing the TCR activation signal throughout the T Cell.

Phospholipase C-γ is one of the most prominent signalling molecules to be activate. PLC-γ interacts with both LAT and Slp76 in the complex and progresses the signal through the hydrolysis of phosphatidylinositol bisphosphate(PIP_2)(72). PIP_2 is hydrolysed to produce diacylglycerol(DAG) and inositol triphosphate(IP_3)(73). Both DAG and IP_3 have an important role in downstream signalling.

DAG recruits and activates multiple proteins, the most important of which are Protein Kinase C-θ(PKC-θ) and Ras activating guanine nucleotide exchange factor(RasGRP)(74). RasGRP activates the Ras protein, therefore initiating the MAPK/ERK pathway. This pathway exerts many positive effects on the cell such as: stimulating proliferation through the activation of c-MYC and ELK-1; encouraging cell survival through the activation of RSK, which activates CREB whilst inhibiting BAD decreasing apoptosis; and inhibiting BIM further suppressing apoptosis(75). The signalling additionally promotes the transcription of NF-kB transcription factor. NF-kB controls multiple cellular processes overall inducing a cellular survival and proliferative response(76).

Alternatively, Ca2+ signalling is induced by IP3. IP3 causes the release of Ca2+ ions from the endoplasmic reticulum into the cytoplasm which stimulates the opening of calcium release-activated cell Ca2+ channels within the cell surface membrane causing an influx of Ca2+ into the cell(77). The high concentration of Ca2+binds to calmodulin activating calcineurin(78). Calcineurin is a serine/threonine phosphatase that promotes the transcription of the IL-2 gene via nuclear factor of activated T cells(NFAT). The up-regulation of IL-2 is able to further induce T cell growth and differentiation causing an expansion of the antigen specific cytotoxic T Cells enhancing the immune response.

1.4.4 Tumour specific T Cell Priming

Developing tumours are often highly immunogenic due to the creation of novel and altered proteins. These proteins have the capacity to induce a specific immune response against the tumour. Experimental evidence, using EL4 cell line transduced with an NP366 epitope as a novel antigen, demonstrated that following inoculation there was a significantly high prevalence of NP366 specific CD8+ T cells detectable in both the peripheral blood and tumour draining lymph nodes within 6 days, followed by the spleen in 9(79). Further patient studies have shown that it some cases of metastatic melanoma up to 10% of peripheral circulating CD8+ T cells are specific for a single tumour associated antigen(80).

The development of such a specific CD8+ immune response has been believed to occur due to antigen presentation within the lymph node by professional antigen resenting cells such as DCs. However, it is noted that often a strong tumour specific T cell response is only seen in the presence of metastatic infiltrates that have migrating into the lymph node, suggesting a role for the cancerous cells, themselves, in induction of a specific immune response(81). The observation of metastatic induction of CTL responses lead to the proposal of two alternative mechanisms of induction.

The classical pathway of tumour specific CD8+ T Cell induction involves professional antigen presenting cells, which an endocytose the whole cancer cell or tumour associated proteins. The cellular proteins are then degraded in the endosome before being bound to an MHC molecule and transported to the cell surface membrane, by a protein named TAP(82). The APCs then home to a secondary lymphoid organ, such as the lymph nodes, where they present the degraded peptide to naive T cells inducing activation and proliferation of a tumour antigen specific T cell response. This mechanism is referred to as cross-presentation, due to the requisition of an intermediate cell. However, experimental models have concluded that this is not the sole mechanism.

Genetically modified TAP deficient mouse models produce an experimental model that is unable to facilitate cross priming as the APCs are not capable of transporting the endocytosed peptide from the cytosol to the endoplasmic reticulum, inhibiting binding with the MHC complex

and therefore cell surface presentation. Naive NP366 specific monoclonal T cells were adoptively transferred into circulation before NP366-EL4 tumour inoculation. 2 days following inoculation the tumour draining lymph nodes clearly showed a high presence of tumour associated antigen specific CD8+T cells, confirming an alternative and direct pathway of immune induction by cancer cells in absence of efficient APCs.(79) It is therefore most likely that metastatic cells are able to migrate to the lymph nodes, directly priming and inducing tumour specific CD8+ immune responses.

1.4.5 Alternative Co-stimulation

The classical two signal model of naive CD8+ T cell activation involves cognate interaction of the TCR with MHC1 bound foreign antigen, proceeded by a secondary signal via a co-signalling receptor. TCR signalling alone leads to T cell anergy as well as sustained levels of p27, a cell cycle blocker(83). Priming of CTLs by professional antigen presenting cells is the primary mechanism of T cell stimulation. APCs express multiple cell surface molecules capable of providing the secondary(co-stimulation) signal for efficient T cell activation, the most studied of which are CD80 and CD86. CD80/CD86 both interact with CD28 on the T cell surface, causing its co-localisation to the TCR(84). This clustering stimulates the formation of the cSMAC and pSMAC, creating a sustained immunological synapse and therefore initiating downstream T cell signalling(85). Many malignancies down regulate the expression of CD80/CD86 as an immunological evasion mechanism, resulting in what would deem a cold tumour.

However, many tumours can still provide two distinct T cell activation signals, in the absence of CD28(86). In the absence of CD28 the second stimulatory signal is achieved through the utilisation of alternative co-signalling molecules. Studies have described an array of alternative co-signalling molecules, as seen in figure 4, capable of both initiating(co-stimulating) and suppressing(co-inhibiting) complete T cell signalling(87). It is these co-signalling receptors that determine the ultimate differentiation fate, proliferation and survival of the T Cell. ICAM-1 is a co-signalling receptor of the immunoglobulin superfamily. Primarily identified for its role in leukocyte

arrest and extravasion, ICAM-1 binds to LFA-1 on leukocytes, halting them from their capillary rolling mechanism and enables extravasion from the blood into the surrounding tissues. Recent studies have further explored the multifaceted role of ICAM-1 and have determined a mechanism by which ICAM-1 can provide the secondary co-stimulation signal, through binding of LFA-1, in turn initiating T cell signalling(88). Binding of ICAM-1 to LFA-1 on T cell surface causes activation of PI3K, enabling T Cell proliferation, increased IL-2 secretion and rates of proliferation following co-stimulation are comparable with that following classical CD28 co-signaling(89). Paradoxically, the number of cells that undergo three or more divisions is seen to decrease 3-fold. In addition, LFA-1 co-stimulation has been shown to cause a significant decrease in p27, but that the remaining level was consistently slightly higher than with CD28 co-signaling(90). This suggests that LFA-1 signalling can induce a significant T cell response however that the response may not be as efficient or sustained as that of classical CD28 signalling. In contrast, an alternative study discovered an increased percentage of cells undergoing proliferation post ICAM-1 co-stimulation(75%) compared to CD28 co-stimulation(55%) whiten 72hrs(91). Therefore, implying that the paths of co-stimulation may not cause a significant difference in cellular proliferation.

Experiments using the renal carcinoma cell line(Renca), have demonstrated that signalling via ICAM-1 LFA-1 interactions are able to of induce a tumour specific immune response. In vitro malignant renal carcinoma(Renca) cells, expressing the influenza virus(A/PR8/H1N1) haemagglutinin antigen(HA) as a neo-tumour specific antigen, demonstrated the ability to induce significant CD8+ T cell proliferation upon co culture with naive HA specific monoclonal T Cells(CL4)(92). Renca cells lack expression of the classical co-stimulatory molecules CD80/CD86, however do express ICAM-1. This in vitro study confirmed a possible mechanism for direct tumour cell T cell activation utilising alternative co-stimulatory mechanisms.

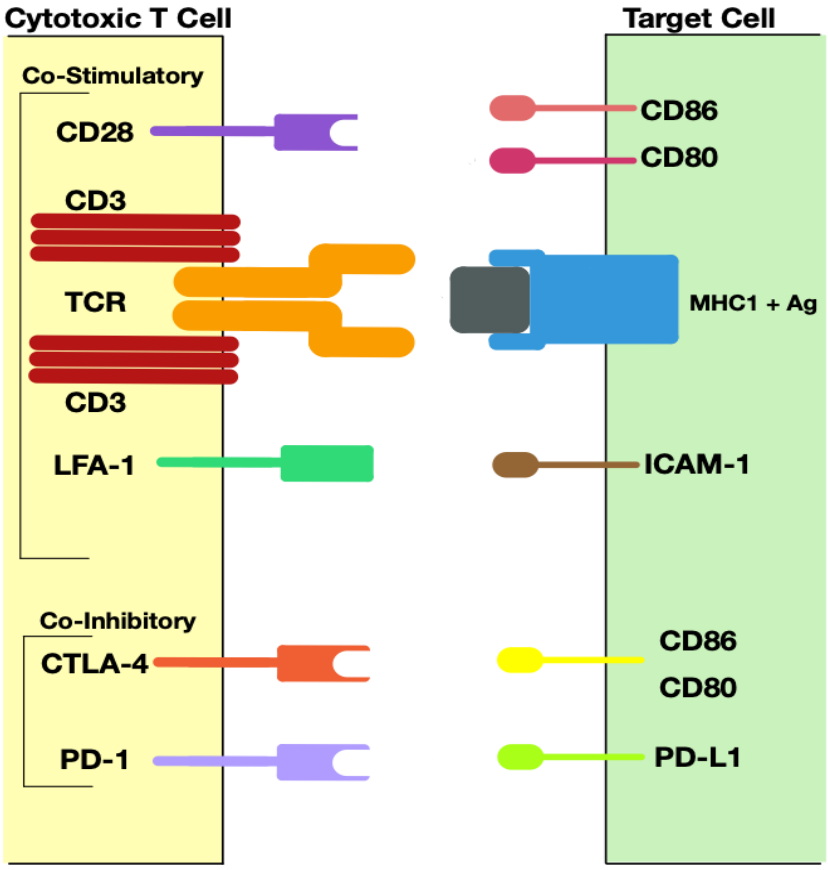


Figure 5: Cytotoxic T Cell Co-signalling molecules

The balance of co-signalling molecules ultimately decide the fate of interacting CTLs. The classical two signal model of naive CD8+ T cell activation involves cognate interaction of the TCR with MHC1 bound foreign antigen, proceeded by a secondary signal via a co-signalling receptor. Co-stimulatory CD86 and CD80 bind either CD28 inducing CTL co-stimulation or its competitive co-inhibitory receptor CTLA-4. More recently, co-stimulation via LFA-1:ICAM-1 interaction has been demonstrated to effectively stimulate T cell signalling. PD-L1 binds to the co-inhibitory receptor PD-1, suppressing T cell function.

1.4.6 CD8+ T cell cytotoxic function

CD8+ T cells are key mediators in the adaptive immune system, with a primary role of eliminating compromised cells infected with intracellular pathogens such as bacteria and viruses. Additionally, they hold a vital role in eliminating transformed cells, therefore preventing the development of cancer. Cytotoxic CD8+ T cells have three main cytolytic mechanisms, all of which utilise the activation of apoptosis. These include: the release of cytokines, FAS induced apoptosis, and the release of cytolytic granules.

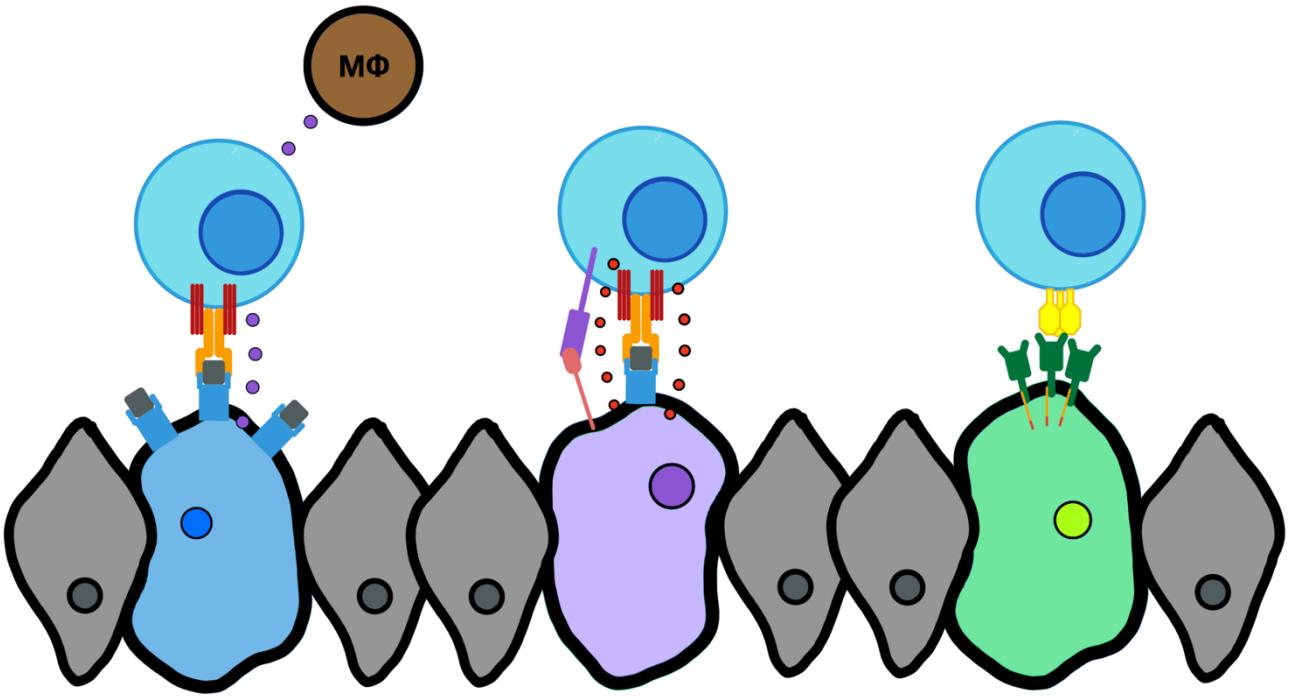


Figure 6 : Mechanisms of CD8+ T cell cytolysis

The primary mechanisms of direct CD8+ cytotoxicity is by the polarisation and release of cytolytic granules containing perforin and granule-associated proteases collectively named granzymes. Perforin and granzymes are essential for efficient elimination of infected cells and are therefore stored into modified lysosomes forming lytic granules(93). This method of storage ensure they are inactive when inside the T cell preventing damage. Upon recognition of a MHC1 bound antigenic peptide, via the T Cell receptor, rearrangement of internal actin cytoskeleton occurs. Cytoskeletal rearrangements cause a migration of the microtubule organising center(MTOC) towards the forming immunological synapse(94). Binding between CD28 and CD86/CD80 and/or LFA with ICAM-1 stabilise the immunological synapse to enable sufficient time for cytolytic activity. Succeeding the MTOC, the lytic granules subsequently polarise towards the cell surface membrane causing docking and release into the synapse by exocytosis(95). Formation of a stable immunological synapse preceding the release of granules is essential to ensure the perforin and granzyme are directed, in high concentration, solely at the target cell.

Initial studies suggested perforin was believed to create pores in the target cell surface membrane enabling granzyme to efficiently diffuse into the cell inducing cell death. However, more recent studies have suggested that a more likely mechanism is the endocytosis of both molecules. Perforin is believed to then form pores in the endosome mediating the release of granzymes into the cytoplasm of the target cells, inducing cell death(96). The two primary granzymes released by CD8+ T cells are: Granzyme A and Granzyme B. Some studies have suggested that as proteases it is possible that granzymes can act in both intra and extracellular fashion. In vitro studies have demonstrated their ability to cleave extracellular matrix proteins, such as fibronectin, inducing the release of cells and therefore death by anoikis. However, it is well established that their primary function occurs once they have reached the cytoplasm of target cells. Once in the cytoplasm, granzymes can initiate cell death through a variety of mechanisms. The proteases induce apoptosis of the target cell through caspase dependent and independent mechanisms.

Granzyme B induces apoptosis using two main mechanisms. The primary mechanism is the induction of mitochondrial pathway of apoptosis. Granzyme B cleaves Bid, a BH-3 homologous pro-apoptotic protein. Truncated Bid migrates to the mitochondria membrane where it binds to the trigger site of pro-apoptotic Bax, directly activating it, resulting in the release of cytochrome c(97). Cytochrome c binds to an APAP-1 monomer, inducing a conformational change that results in the oligomerisation and formation of the Apoptosome. The Apoptosome activates the initiator caspase 9 which, in turn, activates multiple effector caspases(98). Overall this mechanism induces the condensation of chromatin, laddering of DNA and membrane blebbing, otherwise known as apoptosis. Moreover, granzyme B is in-fact capable of directly activating the initiator caspase, caspase 8, as well as the effector caspase, caspase 3.

In contrast granzyme A's primary cytotoxic mechanism is through the direct induction of DNA single strand breaks(99). An accumulation of single strand breaks can be extremely toxic to a eukaryotic cell especially a cancerous cell due to the potential loss of one or more aspects of the DNA damage response. In addition, granzyme A initiates the permeabilisation of mitochondrial membrane, in turn, causing the release of reactive oxygen species(ROS)(100). Reactive oxygen species are highly reactive oxygen atoms or molecules that are able to interact and damaging a multitude of proteins, resulting in loss of membrane integrity and cell death.

CTLs can also carry out cytolysis via alternative mechanisms such as utilising their expression of the Fas ligand, initiating the "Death receptor pathway"(101). CD8 T cells express the trimeric ligand, Fas, which can act on the Fas receptor(CD95/APO1) expressed by the majority of the cells in the body. The Fas receptor is made up of an extracellular Fas domain and an intracellular "death domain". In the presence of the Fas ligand, Fas receptors trimerise leading to the recruitment of the Fas associated death domain. FADD is an adaptor molecule with homology to the intracellular death domain of the Fas ligand as well as to the death effector domain attached to Caspase 8 and 10 pro-enzymes(102). These pro-enzymes exhibit zymogen function. Due to the clustering of receptors and therefore caspase pro-enzymes, their intrinsic caspase activity enables cleavage of corresponding pro-enzymes releasing fully functioning caspases.

Caspase 8 is then able to exhibit two separate actions. Primarily it uses its caspase activity to activate the executioner caspase 3, inducing cellular apoptosis. In many cells, "Type 1", this pathway is sufficient to kill the host cell. However, in some cells such as hepatocytes, named "type 2", not enough caspase 3 is activated through the primary pathway therefore the active caspase 8 that is produced harnesses the mitochondrial pathway of apoptosis(103). Caspase 8 cleaves the Bid protein causing a conformational change truncating the protein. tBid is then able to initiate the mitochondrial pathway of apoptosis as previously described.

Another indirect mechanism in which CD8+ T cells eliminate intracellular pathogens is by the release of specific cytokines(104). Upon recognition of a foreign MHC1 bound peptide, CD8+ T cells release three key cytokines that enhance further elimination. IFN-y inhibits viral replication within the cell depleting the viral load, whilst also encourages and increased expression of MHC class 1 molecules. This, in turn, enhances the presentation of the viral peptides increasing the immunogenicity of the virus infected cells and enables more efficient recognition and killing by additional immune cells.

Furthermore IFN-y acts by activating and recruiting macrophages to the site of infection, increasing the chance in which the infected host cell will be recognised as a target and undergo cytolytic action(105). TNF-a and TNF-b both work alongside IFN-y to enhance the activation of local macrophages, increasing antigen presentation and cytotoxicity. In addition, TNF-a can act directly on a specific membrane bound receptor, TNFR, that can also induce cellular apoptosis by the Death receptor pathway. Therefore not only do these cytokines allow for increased local killing and enhance CD8+ effector function, but can also directly induce cell death.

1.5 Immunological synapse

1.5.1 Immunological synapse

For efficient interactions between immune cells and their targets to be completed they must form a tight stable interface, described as the immunological synapse. The formation of a synapse is essential before the initiation of specific targeted interactions. CTLs play an essential role in eliminating cancer cells, primarily through the release of high toxic cytolytic granules. In order to achieve target specific killing, limiting co-lateral damage, an immunological synapse must form between the CTL and target before granule release. Upon recognition of a target, stimulation of the TCR induces a reorganisation of the cytoskeleton, as shown in figure 7.

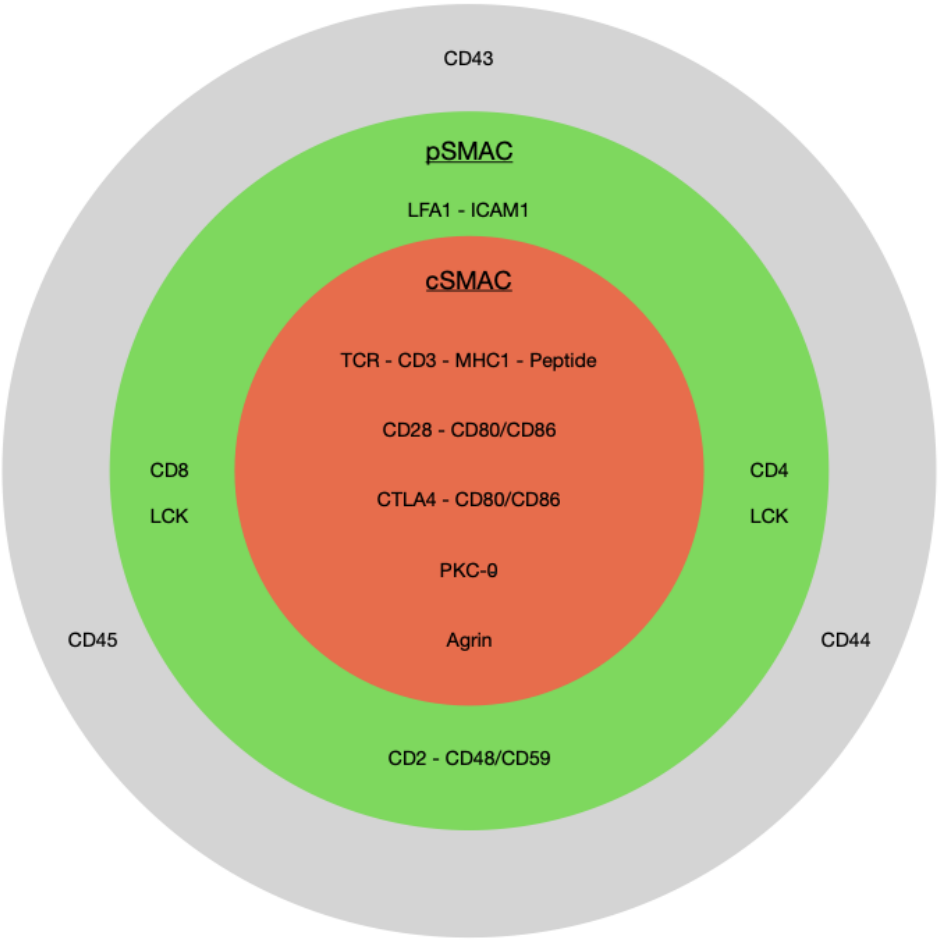


Figure 7: The Immunological Synapse

The eukaryotic cell cytoskeleton consists of tubulin, actin and intermediate filaments. Actin is a protein that can either occur singularly as globular actin(G-Actin) or as part of a polymer as filamentous actin(F-Actin) which plays a key role in the stabilisation of the immunological synapse by forming a peripheral ring structure to stabilise the contact. An accumulation of F-Actin is seen to accumulate at the center of the contact, proceeding cellular interaction. However, the accumulation of F-Actin is seen to quickly dissociate, through the enzymatic action of Cofilin, and instead form a distal ring surrounding the forming SMAC(105). The stable peripheral actin ring enables TCRs to undergo clustering at the center of the interface, alongside key co-signalling molecules, such as CD28, forming the cSMAC. Surrounding the cSMAC, adhesion molecules, such as LFA-1 and the adaptor protein Talin, form a tight ring further stabilising the immunological synapse(106). This structure is named the pSMAC and is tightly surrounded by the stable peripheral actin ring as well as additional proteins, overall forming what has been described as a bullseye configuration(107).

Once formed, the microtubule organisation center can relocate proximally from behind the nucleus towards the synapse(108). Studies have shown that migration of the MTOC holds a critical role, as one of the inner MTOC centrioles docks the cell membrane adjacent to the TCR micro-clusters(109). Stored lytic granules, containing perforin and granzymes, are found associated with the migrating MTOC.

Therefore, the cSMAC contact allows for an extremely concentration and directed release of lytic granules into the immunological synapse exhibiting a cytotoxic effect on solely the target cell through central sustained localisation of the MTOC and release of lytic granules. Additionally it is speculated to focus the admission of the FasL within the synapse further directing cytolytic effects(110).

1.6 Immunotherapy

1.6.1 Conventional vs Immunotherapy

For many years cancer therapy has relied upon three treatments: Surgery, chemotherapy, and radiotherapy. Surgery is used to remove the main tumour mass, whereas chemotherapy and radiotherapy were used to kill cancer cells based on their ability to rapidly proliferate. These treatments have shown impressive results and are still regarded as the forefront of cancer treatment however not without cost. Agents eliminating rapidly dividing cells also dramatically affect other replicating tissues within the body, especially within then blood and immune system. One potential alternative or adjuvant therapy to assist combating cancer without significantly harming the patient is immunotherapy.

Immunotherapy is the enhancement of a patient's own immune system in attempt to combat cancer. The two primary classes either attempt to overcome the immune evasion tactics used by tumours such as their immunosuppressive microenvironment, or generally amplify stimulation of immune system, enforcing a globally enhanced immune response towards the TAA causing specific tumour killing and regression but also potentially host cells.

1.6.2 Non-specific Immunotherapy

The first cytokine immunotherapy was officially approved by the FDA as a treatment against human metastatic melanoma and metastatic kidney cancer. IFN-α had undergone extensive murine pre-clinical trials to finally be sanctioned for its use against Hairy cell leukaemia, a rare form of B Cell leukaemia(111). IL-2 has since been approved for both metastatic renal carcinoma, in 1992, and advanced melanoma, in 1998(112). Cytokine treatment continues to prove great promise as an adjuvant cancer immunotherapy. Cytokines are soluble immune mediators that act both in paracrine and autocrine fashion. They are both produced and act on immune cells, controlling many active biological processes such as cell growth, proliferation and cell death. Cytokine therapy

aims to enhance the immune system in an attempt to drive anti tumour responses. Clinical trials using cytokine therapy against cancer have shown success, enhancing complete remission following treatments of either single agents, IFN-a, IFN-y, IL-2 and TNF-a, or even a mixed cocktail of multiple(113). However, as cytokines only act over a short distance, the intravenous concentration delivered for effective treatment is extremely high. This high concentration has been seen to have the potential to cause dramatic, life threatening side effects such as capillary leak syndrome and neutropenia(114). Due to these side effects and the high dose needed cytokines are not yet commonly used as monotherapy but instead as adjuvant to enhance the potential of tumour specific immunotherapies.

1.6.3 Tumour specific immunotherapy

The class of immunotherapies aimed at enhancing the tumour specific immune response consist of treatments that aim to overcome the decreased immunogenicity utilised by tumours to evade the immune system, in turn increasing specific tumour killing. CD8+ T cells capable of recognising tumour associated antigens are suppressed by numerous mechanisms found in the TME. Many treatments such as PD-1 blockades are currently being trialled and developed that target and inhibit these suppressive mechanisms(115).

Currently one approved active immunotherapy utilises dendritic cells(DC) as vaccines. DCs are essential for the priming of naive T cells. Circulating DC progenitors are isolated on their CD1c+ expression and eluted from a patient's blood sample before being expanded ex vivo using cytokines and growth factors(116). Once an adequate number has been obtained, the DC are peptide loaded with tumour associated antigen and matured via additional maturation signals. Following maturation, the DCs are then reintroduced into the patient's circulation. Introducing a high number of mature, TAA presenting DC cells into a patient's circulation greatly enhances the cross priming of CD8+ T cells, in turn stimulating a greater tumour specific immune response. Dendreon has developed an effective vaccine, through the ex vivo expansion and enhancement of DC cells

presenting specific TAAs(117). Promising results has been seen, one trial showing a 2-fold increase in survival rate over 36 months (118). Currently a number of clinical trials are underway, however so far only one DC cell vaccine, Provenge®, has been officially licenced by the FDA for use in prostate cancer.

Further studies have investigated the possibility of using alternative cell based treatments. One study showing great promise being adoptive T cell transfer. Adoptive T cell therapy uses either peripheral T cells in circulation or isolated tumour infiltrating lymphocytes directly from patient tumour biopsies(119). Tumour infiltrating lymphocytes(TILs) have the innate ability to recognise tumour associated antigens. Tumour biopsies are fragmented and plated in the presence of IL-2, inducing TIL proliferation. TILs are assayed for their ability to recognise tumour-associated antigens, before being expanded ex vivo. Once expansion is complete TILs are reintroduced into circulation. Using IL-2 as an adjuvant has shown to significantly increase the in vivo persistence of TILs following infusion(120). The immense increase in CTLs capable of recognising tumour-associated antigen exerts a significant specific cytolytic effect against tumours. However, even though this treatment enhances the immune system in a specific manner, some side effects are still seen such as vitiligo and uveitis(121). Currently, clinical trials utilising autologous TILs in adoptive T Cell transfer against metastatic melanoma have shown substantial clinical responses in approximately 40% to 72% of patients(122). However, the primary limitation of TIL ACT is the necessity of a pre-existing population of TILs. To overcome this, many studies have been investigating the development of genetically engineered peripheral blood T cells.

T cells may be isolated from peripheral circulation and cultured, as previously described, before being genetically modified to recognise specific TAAs. Genetic modifications are most often completed using engineered replication incompetent y-retroviruses and lentiviruses(123). Viral infection incorporates specific genes into the host genome that enable expression of high affinity

TAA specific TCRs. Specific tumour associated antigens can be subdivided into several categories, seen in figure 8.

Type of TAA:	Example:
Mutated Proteins(Point mutations/Gene rearrangements)	BCR-ABL
Cancer Testis Antigen (Aberrant expression of proteins usually only found in male germ cells)	MAGE, NY-ESSO-1
Differentiation antigens	Melanin
Overexpressed proteins	Her-2/Neu
Abnormal post translational modifications	Aberrantly N-acetylglucosamine modified proteins
Novel proteins	Point mutations/truncations
Viral Oncogenes	HPV 16

Figure 8: Classes of tumour associated antigens

Different classes of tumour associated antigen along with reported examples. The identification of tumour specific antigens, enables targeted CTL responses against solely tumour cells, preventing local cell damage. Tumour specific molecules stem from either mutated proteins, due to genetic instability, which are therefore not expressed in normal tissues, or the aberrant expression of normal functioning proteins by alternative cells.

The genetic modifications generate high numbers of tumour specific CTLs that may then be introduced into circulation. Impressive results have been noted in clinical trials combatting metastatic melanoma as well as oesophageal and synovial sarcomas(124).

Induction of tumour specific TCRs show great promise but is restricted by its ability to only interact with specific MHC1 haplotypes(125). In attempt to overcome the restriction, developments on this theory have evolved into creating further tumour specific CTLs that are able to act in an MHC-1 independant manner. MHC-1 independency has been achieved by the creation of a novel receptor type deemed chimeric antigen receptors(CARs). Currently three generations of Chimeric antigen receptors have been developed, each with an increasing level of complexity and cytoplasmic signalling domains. Chimeric antigen receptors each have an extracellular domain comprised of a single chain antibody or ligand specific for a receptor or protein expressed solely on cancer cells(126). The development of CARs allows for the unrestricted fabrication of genetically modified

tumour specific CTLs, increasing the availability of adoptive T cell transfer cancer immunotherapy. Currently CAR therapy has seen impressive clinical success, with up to a 90% remission, against acute lymphocytic leukaemia(ALL) through targeting CD19 as well as against Neuroblastoma by targeting GD2(127).

Recent developments of soluble versions of genetically engineered high affinity TCRs are generated and bound to an anti-CD3 antibody fragment(128). Anti-CD3 antibodies are capable of cross linking CD3 molecules associated with peripheral CD8+ T cells inducing TCR signalling, named ImmTacs®. Therefore ImmTACs® are able to specifically attach to cancer cells enabling the activation of non-specific peripheral CD8+ T cells, inducing a cytolytic response. This novel technology is primarily still currently under development, however the first clinical trial is underway utilising PO-418 ImmTAC® molecules to target the HLA-A*0201/gp100 expressed on melanoma cells(129).

Despite the rapid development of tumour specific immunotherapies that are showing great promise in the clinic, there are numerous side effects have been identified. Impressive developments have been seen in detecting and generating responses against tumour specific antigens however the overwhelming multitude of immunosuppressive factors produced and expressed by the tumour microenvironment are still proving a problem to overcome.

1.7 Tumour Microenvironment

1.7.1 Tumour microenvironment

Recent advancements in cancer research have led to the identification of a correlation between an increased number of lymphocytes within the tumour mass and a better prognosis following surgery or immunotherapy(130). Lymphocytes that have left the blood stream, migrating into a tumour mass can be identified in tumour biopsies and deemed “tumour infiltrating lymphocytes”. Both B and T lymphocytes are described to infiltrate into both the surrounding stroma and immersed

within tumour mass itself. It is known that tumours commonly down regulate cell surface molecules in attempt to decrease their immunogenicity yet the presence of these lymphocytes within the tumour implies that they have the unique ability to able to recognise a specific tumour associated antigen and therefore to eliminate specifically tumour cells. However, it has been discovered that TILs express a suppressed phenotype, decreasing their cytolytic capabilities(131). A vast amount of research has undergone to identify specific mechanism utilised by tumours to not only evade but actively suppress the immune system.

1.7.2 Inhibitory receptors

One immunosuppressive mechanism utilised by cancer cells is the up regulation of immune blockade ligands. Immune blockade receptors are expressed by active CTLs to modulate their effect function. CTL stimulation is controlled by the balance of co-stimulatory and co-inhibitory receptor signalling, ultimately determining the fate of the cell. This enables the tight regulation of CTL effector function, reducing the risk of unnecessary bystander effects or autoimmune disease. Upon reoccurring exposure to antigen CTLs face exhaustion and therefore up-regulate immune checkpoint receptors, predominantly Programmed Death Receptor-1(PD-1) and CTLA-4(132).

PD-1 is a co-inhibitory receptor, from the CD28 family, known to be expressed on a plethora of immune cells but it primarily recognised for its role in modulating active CTL effector function. Upon recognition of a target MHC1 complex CTLs release inflammatory cytokines, increasing the local expression of PD-L1 as a tolerance mechanism to prevent damage to local bystander cells(133). It is mainly activated through the recognition of the PD-1 Ligand(PD-L1), however reports have described a possible mechanism by which it is also stimulated through CD80, similarly to CTLA-4(134). Upon binding PD-1 exerts an inhibitory effect, causing T cell suppression and dysfunction, the mechanism by which is still largely unknown. However, it has recently been proposed to disrupt the maintenance of the immunological synapse(135).

Several cancers have been shown to exploit this mechanism by expressing high levels of PD-L1 on their cell surface(136). Additionally, up regulation of PD-1 expression seen on tumour infiltrating lymphocytes suggests the TME has a role in driving this suppressed phenotype. However, identification of this has led to immense immunotherapy developments, with PD-1/PD-L1 blocking antibodies showing impressive clinical success, as previously described.

CTLA-4 is also a co-inhibitory molecule, expressed by activated CTLs, that acts as an immune checkpoint protein(137). CTLA-4 competitively binds CD86/CD80 with an increased affinity to CD28 preventing co-stimulation, whilst additionally actively inducing inhibitory signals. The mechanism of CTLA-4 is still widely unknown, although it has been suggested that it implements a phosphatase action in the cSMAC deterring any induced TCR signalling(138). CTLA-4 is also found to be unregulated upon CTLS entry into the TME, demonstrating its potential for immuno-therapeutics(139). Ipilimumab has shown incredible clinical success especially in the fight against metastatic melanoma, but has also been approved for its use against multiple cancer types(140).

Recent advancements within the field of immunology has led to the discovery of one possible solution, Immune blockade receptor inhibitors. The 2018 noble prize in medicine was awarded for the discovery of two key immune blockade molecules PD-1 and CTLA-4 and their potential for inhibition(141). PD-1 and CTLA-4 are both co-inhibitory receptors expressed solely on activated CTLs. Modulation of T Cell function occurs through the ratio of co-stimulatory and co-inhibitory molecules. Immune blockades primary function is to modulate immune reactions to prevent both over stimulation of CTLs limiting off site side effects, as well as autoimmune disease(142). However immune blockade ligands are found to be highly expressed on cancer cells, as a mechanism of immune evasion. To counteract this, research has developed monoclonal antibodies that target and block these inhibitory receptors preventing immune suppression. This treatment proves to be one of the most promising advancements in cancer therapy, showing clinical success for metastatic cancers with a previously very poor prognosis e.g. metastatic melanoma. Currently two treatments have

been approved by the FDA for use in clinic as cancer immunotherapies: inhibiting antibodies of either/both PD-1(Nivolumab) and PD-L1(Atezolizumab/Durvalumab), as well as blocking antibodies of CTLA-4(Ipilimumab). All of which have shown great clinical success and approval for the treatment of multiple cancer types. Further research is still underway to advance developments against alternative aspects of the tumour microenvironment.

1.7.3 Soluble immunosuppressive factors

Following the success of immune blockade receptor therapies, more and more research has been undertaken to investigate the immunosuppressive effects of the TME. One key category of identified factors are soluble suppressive molecules, including Adenosine and Prostaglandin E2. Adenosine is a naturally abundant purine nucleoside that has an important role in cellular metabolism and signalling(143). It binds to 4 independent G coupled receptors(A1,2a,2b,3), subclassed on their juxtaposed ability to either increase of decrease intracellular cAMP. Receptors A1 and A3 both inhibit the enzyme adenyl cyclase therefore suppressing intracellular levels of cAMP. In contrast, A2a and A2b both increase the activity of adenyl cyclase driving the up regulation of cAMP, which in turn has an extremely suppressive effect on immune cells(144). In the context of CTLS, increased cAMP is capable of suppressing TCR signalling, as well as down regulating the expression of key proliferative and survival transcription factor NF-kB(145).

Hypoxia is commonly associated with the growing tumour. As the transformed cells proliferate uncontrollably, they begin to outgrow the available blood supply leading to a necrotic core, of cells in which oxygen cannot reach through simple diffusion. These hypoxic conditions are believed to be a major driver of extracellular ATP. In addition to this Hypoxia induced factor-1(HIF-1), a transcription factor induces the expression of adenosine producing, ectoenzymes CD39 and CD73. Therefore, ATP is degraded via CD39 and CD73, creating high levels of adenosine in the TME(146). The increased level of adenosine seen in the TME has been demonstrated to prevent CTL proliferation and activation, whilst inducing T cell anergy function via a A2a dependent

mechanism(147). It is therefore believed that adenosine is a significant contributing factor towards tumour immune evasion.

This identification has led to research of adenosine receptor blockades as a potential adjuvant immunotherapy. However, to date, no therapeutics have made it past preclinical murine models. Instead adenosine producing ectoenzymes, CD39 and CD73 have been used as prognostic markers in multiple types of cancer, including but not limited to ovarian, gastric, colorectal and melanoma(148).

1.7.4 Common immunosuppressive mechanism

Several suppressive mechanisms are believed to operate within the TME, however how individual mechanisms occur, as well as the extent of their overall contribution in suppressing CTLs is still widely unknown. The diverse interactions of inhibitory molecules prove difficult to combat with a single immunotherapy. In addition, cancer itself is a collection of over 200 individual diseases, each with its own concoction of potentially suppressive mechanisms. Therefore, the discovery of a common immunosuppressive mechanism would hold great potential for an effective translational cancer immunotherapy. One possible common immunosuppressive mechanism is the loss of maintenance of the stable immunological synapse. Recent studies have demonstrated a link between the loss of cytolytic ability and the loss of a stable synapse(135). Following tumour infiltration it has been noted that TILs exhibit a decreased ability to maintain a stable immunological synapse.

Firstly, for effective cytolytic effector function the MTOC must localise to the immune synapse bringing with it associated granules for release. Studies have shown upon target cell interaction the MTOC localisation in TILs is significantly reduced(135). Further research demonstrated this to be coupled with an unstable cell couple maintenance. CTL:Target cell couples are well maintained for up to 12 minutes in a symmetrical synapse. However, TIL:Target couples were seen to lose symmetry merely 2 minutes after initial formation, suggesting an unstable interface(135). Coupled with this was the significant increase in off-site lamella formation(135). A CTL lamella is a small

membrane projection controlled by the actin cytoskeleton. They are often seen generated towards the immunological synapse for maintenance of the stable bond. However, TILs exerted a significant increase in off-site lamella, projecting away from the interface and believed to exemplify an unstable interface(135).

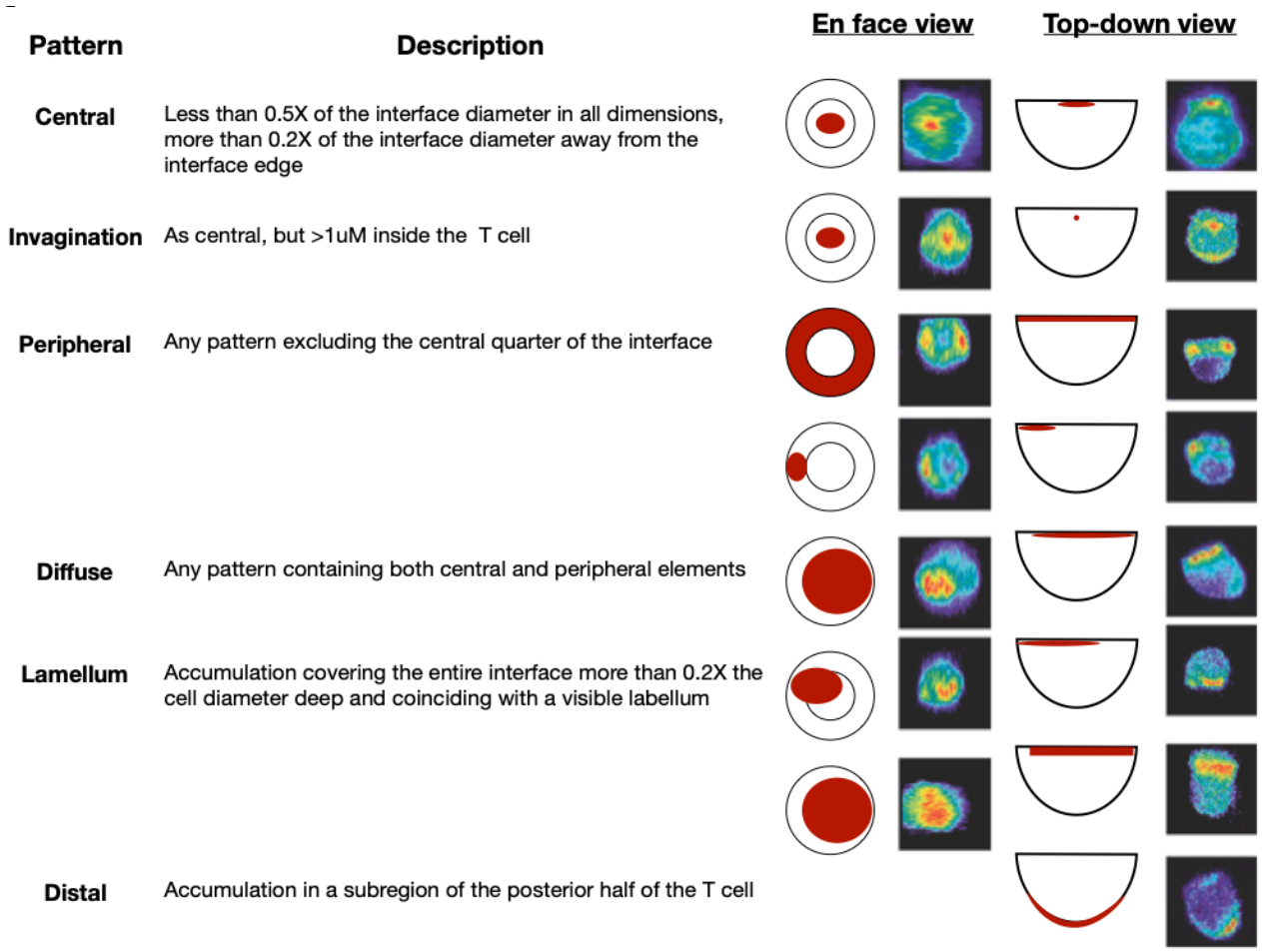


Figure 9: F-Actin Interface Patterning

The immunological synapse is maintained through the formation and maintenance of a peripheral actin ring at the interface in order to stabilise the interaction. Studies have identified alternative actin accumulation patterns indicative of impaired CTL synapse maintenance as an induced suppressed phenotype, following CTL infiltration into the tumour microenvironment. *Adapted from Wuelfing et al, Early Signaling in Primary T Cells Activated by Antigen Presenting Cells Is Associated with a Deep and Transient Lamellal Actin Network, 2015.*

To further investigate the maintenance of the immunological synapse, studies aimed to determine whether the TME had any effect on the intracellular actin polarisation playing a pivotal role in the maintenance of the stable interface. CTLs and TILs were both assessed for their action patterning, as seen in figure 9, as well as the presence of cofilin. Cofilin is an actin binding protein,

that plays an essential role in actin polarisation through its ability of disassembling filamentous actin polymers. Upon host:target cell interactions, peripheral actin ring formations not seen to be significantly affected. However, within 180 seconds a dramatic reduction in sustained peripheral actin ring was seen(135). This effect spanned over 420s with a significant reduction in the percentage of remaining peripheral actin rings(135). The combined data suggests defective intracellular polarisation as mechanism of CTL suppression following tumour infiltration.

To determine whether this phenotype affected the effector function of CTLs, additional research utilised Jasplakinlide treatment to simulate the dysfunction of actin dynamics following tumour infiltration. Jasplakinlide is a potent stimulant of actin polarisation(135). Following treatment, the CTLs showed a matched inability to maintain the peripheral actin ring alongside a significantly reduced cytolytic function. In conclusion this data suggests that dysregulation of actin dynamics and intracellular polarisation exerts an important role in TME suppression and could additionally show potential as a common mechanism of immunosuppression.(135)

1.8 Prostaglandin E2

1.8.1 Introduction

Prostaglandins are lipid mediators involved in a wide variety of biological functions throughout the body. They are as part of the subclass of signalling molecules, the eicosanoids. They are produced by almost all nucleated cells in the body and are found in almost all tissues either constitutively for homeostasis or unregulated due to external stimuli such as trauma and inflammation, signalling molecules, and cellular signalling. All prostaglandins contain a 5-carbon ring and 20 carbon atoms however their structural differences give rise to a wide variety of physiological effects. In humans the most prevalent prostaglandin is Prostaglandin E2(149). PGE_2 itself has been demonstrated to have an immensely diverse physiological effect depending on the tissue, cell type and receptor it binds

to(150). However ultimately its effects are focused around the stimulation of intracellular pathways that result in either the activation or inhibition of adenyl cyclase causing increased or decreased levels of intracellular calcium, respectively.

1.8.2 PGE_2 Production

The synthesis of Prostaglandin E2 involves a multitude of enzymes in a primarily 3 step pathway starting with cell plasma membrane, as seen in Figure 10.

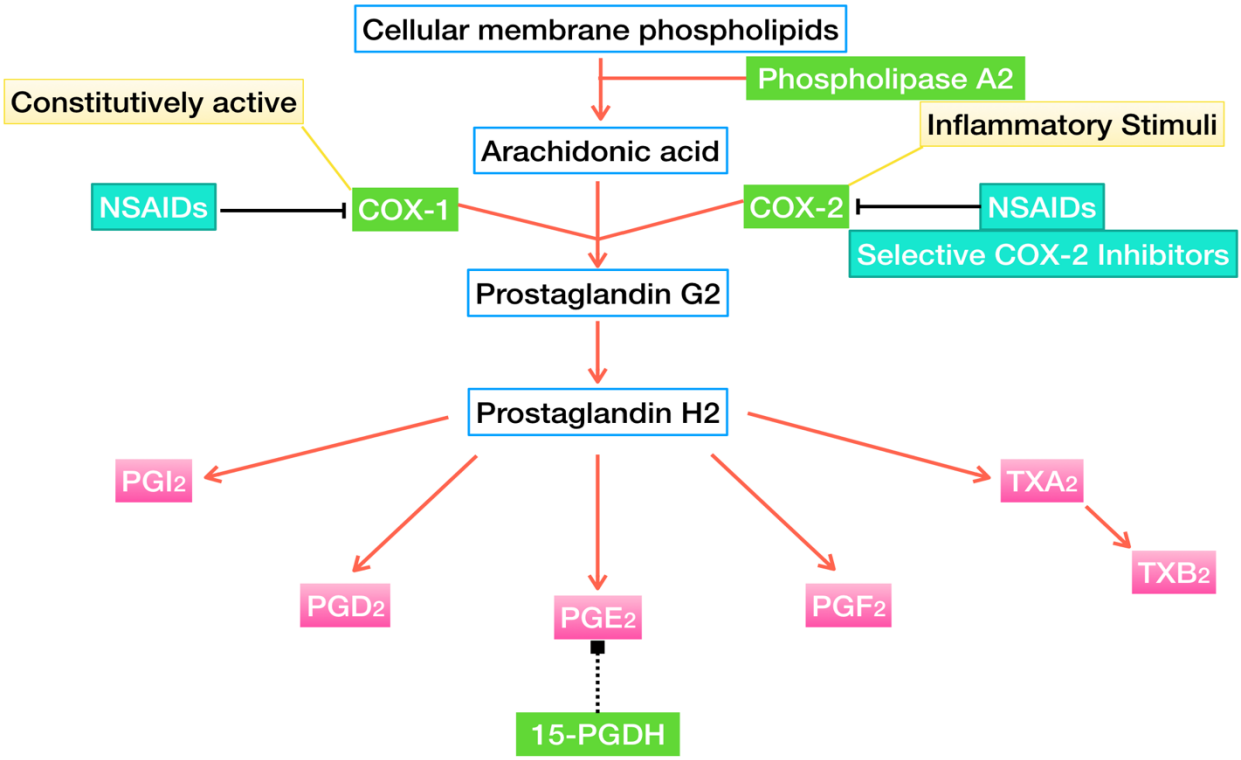


Figure 10 : Prostaglandin E2 synthesis pathway

The cell surface membrane is a bilayer made up of phospholipids. Phospholipids are typically made up of two hydrophilic fatty acid molecules bound to a hydrophilic phosphate group. These fatty acids can be liberated from the phospholipids, via hydrolysis by the enzyme phospholipase A2, in the form of Arachidonic acids(151). Arachidonic acids are comprised of a polyunsaturated 20 carbon chain and are primarily utilised as secondary signalling molecules. In addition to signalling AA is a key precursor in the synthesis of two key lipid signalling molecules: Leukotrienes and prostaglandins. AA are either metabolised by prostaglandin G/H syntheses, collectively named COX1-2, to syntheses prostaglandins or lipooygenases to produce leukotrienes. COX enzymes are bifunctional enzymes that can exert not only cyclooxygenase activity but also peroxidase, distinguishing as two isoforms COX 1 and 2(152).

COX1-2 collectively oxygenate AAs resulting in the production of prostaglandin H2(PGH2). PGH2 can be further metabolised, into 4 key eicosanoids: PGD2, PGE2, PGI2 and TXA2. PGE2 is the most prevalent and well-studied of the eicosanoid family and is synthesised from PGH2 by the enzyme Prostaglandin E synthase(153). Once synthesised PGE2 can act in both paracrine and autocrine fashion until it is degraded by the enzyme 15-Prostaglandin Dehydrogenase(15-PGDH). Homeostatic turnover of PGE2 is typically very quick, ensuring low levels of PGE2.

The level of PGE2 production is controlled by the expression of COX1 and COX2. COX 1 is constitutively expressed in tissues, due to its involvement in tissue homeostasis. In contrast, COX2 is typically expressed at very low, even undetectable, levels but is seen to be unregulated in response to external stimuli such as inflammatory cytokines, as commonly seen in chronic inflammatory conditions, or due to carcinogenesis(154).

1.8.3 PGE_2 Receptors

Part of the complexity of PGE_2 signalling arises from the fact that there are 4 individual receptors and their varied levels expression patterning. Collectively termed EP1-4, EP receptors are serpentine transmembrane G couple receptors that bind PGE_2 inducing distinct signal transduction pathways, as seen in figure 11(155). The pattern of expression of EP receptors widely varies throughout the body depending on the tissue and cell type, however their exact distribution is still largely unknown due to their typically low levels of expression(156).

Each EP receptor consists of a single polypeptide chain that spans the cell surface membrane seven times forming multiple helices, creating an overall barrel tertiary structure. Upon ligand binding the receptor structure undergoes cytoplasmic conformational changes enabling transduction of the intracellular signal via a G-protein coupling mechanism(157). Each EP receptor initiates an independant signalling cascade however their principle mechanisms all engage either the cAMP or PI3K pathways. EP subtypes primarily bind to PGE_2 but each with a different affinity(158). Contradicting studies can be found describing alternative affinities of the EP receptors for PGE_2 in both human and murine models, however most agree that in humans the order is seen to be EP3>EP4>EP2>EP1 contrasting to murine models in which EP3 is still believed to have the

highest followed by EP4 however EP1>EP2(159). The altered expression and affinities between human and murine models further increase the complexity when attempting to investigate the EP receptors role using murine models.

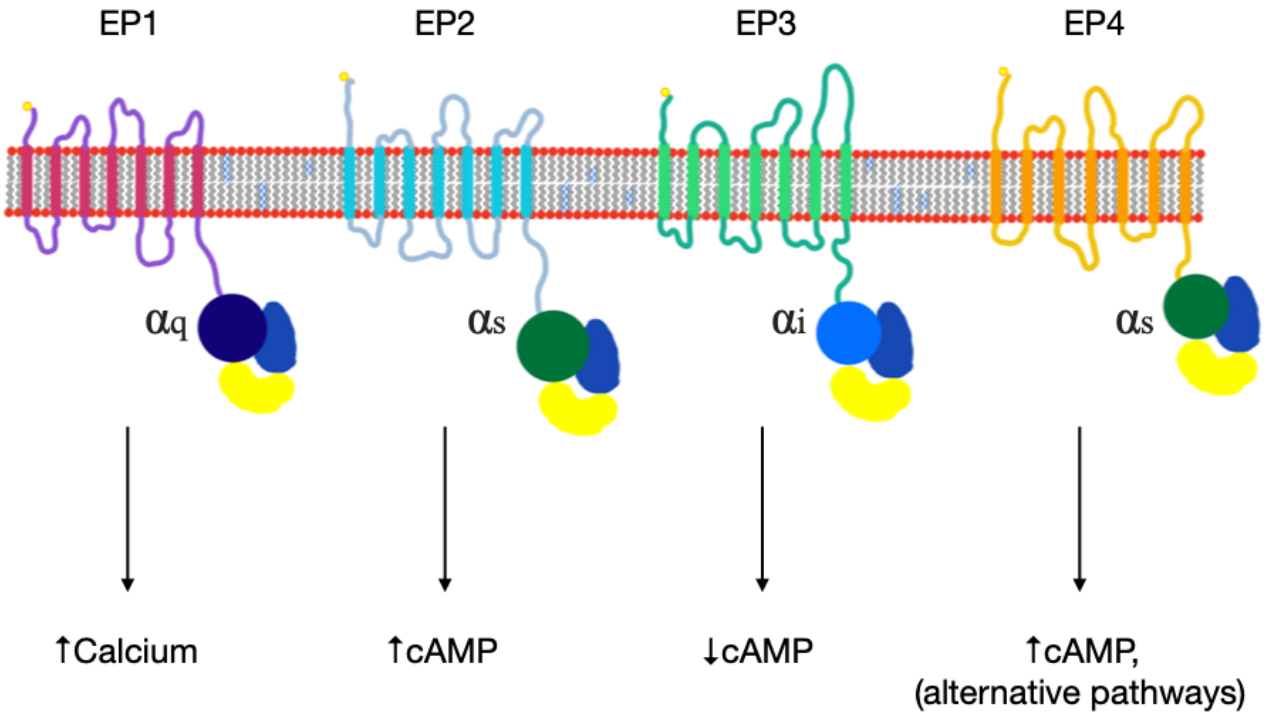


Figure 11: PGE_2 receptors

The EP1 receptor is deemed a 'contractile' prostanoid receptor based to its ability to contract longitudinal smooth muscle(160). Upon ligand binding EP1 undergoes a conformational change causing mobilisation of its Gαq subunit inducing activation of both PLC-y and PI3-k, in turn, through phosphorylation. This action leads to an increase of cytosolic Ca2+ ions, initiating a further influx of Ca2+ via extracellular channels, causing contraction of the smooth muscle. In contrast EP2, EP3 and EP4 receptors exert their action through the inhibition or promotion of cAMP.

EP2 and EP4 share a common pathway through the induction of adenyl cyclase, increasing cytosolic cAMP and inducing Protein Kinase A(PKA), a kinase that can interacts with multiple downstream signalling molecules and transcription factors, such as CREB, altering a variety of gene expressions depending on the type of cell. Upon ligand binding, both receptors undergo an intracellular conformational change causing the dissociation of the $G\alpha_s$-Gβγ complex. The distinguishing difference between EP2 and EP4 is the pathway leading to the induction of PKA.

EP2 acts primarily in a cAMP dependent manner, whereas EP4 also induces PI3-k additionally leading to PKA induction(161). In both EP2 and EP4 receptors $G\alpha_s$ mobilisation, following $G\alpha_s$-Gβγ complex dissociation, activates adenyl cyclase causing increased intracellular cAMP and therefore PKA. In addition, EP4 receptors also induce the PI3k/AKT/mTOR and ERK pathways, as well as inducing Prostaglandin E receptor 4-associated protein(EPRAP), causing suppression of transcription factor NF-kB, an important regulator of inflammation, cellular proliferation and growth(162). The multiple pathways of EP4 contribute to PGE2's multifaceted role in immuno-regulation.

EP3 receptors are deemed inhibitory due to their ability to suppress adenyl cyclase, counteracting both EP2 and EP4(163). Unique to EP3, it has 3 identified isoforms in mice, each comprising of different G protein subunits, that can all exhibit different signalling mechanisms(164). The predominant isoform, $G\alpha_i$-$G\beta\gamma$ inhibits turn suppressing adenyl cyclase, in intracellular cAMP production upon ligand binding. Overall the sheer diversity of PGE_2 receptor expression, tissue patterning and signalling pathways provides a complex obstacle to overcome when investigating the plethora of effects exerted by PGE_2.

1.8.4 PGE_2 in cancer

Altered levels of PGE_2 have been widely noted in a broad spectrum of cancers implying a possible link with tumour development and survival. Studies have demonstrated a significant increase in COX2 expression in multiple tumour types, especially colorectal, renal, lung and mammary(165) Coupled with the increase in PGE_2 synthesis, many tumours have been noted to exhibit suppressed levels of 15-PGDH, decreasing the rate of PGE_2 hydrolysis therefore further increasing PGE_2 levels. As PGE_2 has an immense diversity of effects depending on the stimulated receptor and the cell type, it holds a multifaceted role within carcinogenesis. Multiple studies have linked PGE_2 to all 6 of the original hallmarks of cancer(166). Experiments investigating colorectal cancer have demonstrated a synergistic mechanism of PGE_2 and Wnt signalling(167). Binding of PGE_2 to EP2/4 receptors has the ability inhibit the β-catenin destruction complex, via activation of the cAMP/PKA pathway, stimulating a self-replicative phenotype, further driving carcinogenesis(168). However, PGE_2 does not solely affect tumour cells directly, it also exhibits substantial effects on immune system potentially helping tumour immune evasion.

These effects are especially paradoxical when it comes to the immune system. Typically stimulated by inflammation PGE_2 surprisingly exhibits both pro and anti-inflammatory effects, making the identification of carcinogenic contribution extremely complex. The variation of effects seen on the cells of the immune system largely depend on their level of maturity, the concentration of prostaglandin and interaction with corresponding cells.

Studies discovered a pro inflammatory role for PGE_2 as a potent chemoattractant for Mast cells. Both immature(2 week) and mature(10 week) bone marrow derived mast cells were seen to

express EP3 and EP4 receptors. However, the migratory study, using specific EP3 and EP4 antagonists, clearly demonstrated the chemoattractant mechanism was solely EP3 dependent(169). In addition to attracting mast cells to sites of inflammation, one study has described PGE_2 to greatly enhance mast cell production of pro-angiogenic factors, in a dose dependent manner. Within 2 hours of culture with PGE_2 mast cells were seen to excrete up to 15-fold more monocyte chemoattractant protein-1(MCP-1), a key modulator of monocyte migration and infiltration, further driving inflammation(170).

Similarly, experiments have shown PGE_2 to induce the expression of further chemotaxis mediators from colonic epithelial cells. PGE_2 was seen to induce up regulation in the gene expression and stability of IL-8, a potent chemokine involved in the recruitment of Neutrophils and T Cells, in both a time and dose dependant manner(171). This also enhances local inflammation. Principally there are multiple pro-inflammatory effects of PGE_2, hence its common description as a pro-inflammatory modulator, however in the context of cancer PGE_2 is primarily mentioned for its immunosuppressive consequences.

Research has shown that NK exhibit suppressed cytolytic capabilities, in a dose dependent manner, when in the presence of PGE_2. This is believed to be due to the significant increase in cellular cyclic AMP levels(172). Inhibition of cytolytic action by NK cells enables tumours to develop non-immunogenic phenotypes through down regulation of MHC1 complexes whilst preventing their elimination via NK cell lysis. In addition to a reduction in lytic unit production, a reduction in cytokine production as also seen when NK cells are cultured for 2 days in varied levels of PGE_2(173). A significant reduction of IFN-y production has been noted, believed to be due to IL-15 receptor down regulation again in a dose dependent manner(174).

Contradicting studies have been seen when describing the effect of PGE_2 on FOX-P3 expression in Treg cells. Some studies state that PGE_2 increase the inhibitory function of T reg cells, inducing an enforced Treg phenotype on CD4+ CD25- T cells, as well as causing the up regulation of the FOXP3 transcription factor(175). In contrast, it has also been recorded that PGE_2 signalling,

via the EP2 receptor, lead to stimulation of the cAMP and PKA pathways, negatively influencing the expression of FOXP3 and total Treg differentiation(176). Further research believes to have identified a link between increased PGE_2 expression and expansion of existing Tregs through the promotion of increased interactions with DCs(177).

B cell activation is essential to enforce a full set of functioning immunoglobulins. PGE_2 has been reported to influence early regulatory events in B Cell activation, decreasing the ability to complete efficient immunoglobulin class switching. This is believed to the due to the ability of PGE_2 to effect intracellular cAMP signalling.(178)

Recent studies have demonstrated that the addition of 1uM PGE_2 at the time of CD8+ T cell priming is sufficient to suppress both cellular divisions and IFN-y production. Monoclonal CTLs primed in vitro using Anti-CD3 and rICAM-1 stimulation, used to represent direct priming through lymph node metastases, was seen to be suppressed almost 3-fold by a significant reduction in CTLs reaching their last two divisions(from 75% vs 35%) following addition of 1um PGE_2. Coupled with this was a large reduction in IFN-y production(21.8% vs 9.64%)(91). This phenomenon was seen to be CTL dependant through further investigations, inducing proliferation using irradiated murine renal carcinoma cells. Significant decreases in proliferation were only witnessed when 1um PGE_2 was added directly with the CTLs, in contrast to solely the Renca cells culture, therefore ensuring the PGE_2 was affecting the CTLs directly rather than the Renca cells(179). The immunosuppressive effect was additionally examined and demonstrated to occur through classical co-stimulation. CTLs primed with Anti-CD3 and Anti-CD28 were also shown to exhibit suppressed proliferation upon the addition of 1uM PGE_2, suggesting this suppression would be inclusive to cross presentation(91). This hypothesis was tested on varied levels of PGE_2 to determine whether the response was dose dependant. At 1uM a strong immunosuppressive response was seen, as described, however at lower concentrations(10pM) PGE_2 was seen to exhibit a pro-immune response, increasing the proliferation of CL4 when activated via irradiated tumour cells(179).

Additionally, studies investigated whether the immunosuppressive effect of PGE_2 was reversible once the stimuli was removed. Primary and secondary cultures were set up to determine whether the effect of PGE_2 was sustained. The additional of PGE_2 to primary culture induced a reduction in both IFN-y production ad proliferation. However once environmental PGE_2 was removed for secondary culture, an increased number of proliferation and IFN-y producing CTLs was seen compared to conditions of sustained PGE_2, therefore suggesting that the suppressive effects of PGE_2 were reversible if the stimuli is removed(179). Lastly, cell lysates from the experiment showed a significant increase in intracellular cAMP levels in the PGE_2 induced suppressed cells, suggesting that PGE_2 immunosuppression acts through an cAMP dependent mechanism(179).

Overall the immunological effects of PGE_2 vary vastly, depending on the cell type, the receptors stimulated and concentration achieved. However, studies would suggest that increased levels(1um) of PGE_2, if sustained, induce an immunosuppressive response and could be, in part, responsible for the immunosuppressive TME.

1.8.5 Renca Model

CD8+ T Cells are a key mediator of tumour specific cytotoxicity. CTLs play an integral role in the suppression of tumour development and therefore must be overcome for tumour formation. Many tumour mediated cellular interactions, such as the expression of co-inhibitory receptors and suppressive soluble molecules, have been shown to CTL responses. To examine tumour specific CD8 T cells responses, our laboratory has developed and utilized a transgenic BALB/c murine **re**nal **ca**rcinoma model,(Renca), model in which Renca cells are made to express the haemagglutinin(HA) protein from influenza virus A/PR/8(H1N1) as a neo-self-antigen and can be primed and killed by K^dHA -specific TcR transgenic CL4 CD8 T cells. Studies have showed that PGE_2 is able to decrease proliferation of CL4 T cells in response to RencaK^dHA , due to its ability to reduce IFN-y production which is required to upregulate ICAM-1 expression, which is essential

for priming of naïve CL4 T cells as they express do not express classical costimulatory co-stimulatory molecules CD80 / CD86(91).

1.9 Aims and Objectives

1.9.1 Investigate tumour-mediated suppression by PGE_2

It is accepted that the tumour microenvironment(TME) plays a role in inducing suppressing the CTL activity of $CD8^+$ tumour infiltrating lymphocytes(CTL). Using the Renca / CL4 tumour model, the study aims to determine whether or not PGE_2 is capable of suppressing the effector function of activated CTLs.

Hypotheses:

- PGE_2 inhibits stable maintenance of the immune synapse.
- PGE_2 affects TCR signalling
- PGE_2 suppresses cytolytic function of activated CTLs.
- PGE2 suppresses the proliferation of CTLs

The study aims to identify any alternations to the stability immune synapse as well as the actin patterning, post exposure to PGE_2 for 24hrs, as this time length is representative to the time CTLs spend in the in vitro spheroid model and within the tumour microenvironment when utilised in an adoptive transfer model. The immune synapse studies shall be complete this using confocal microscopy of retrovirally transduced CL4 T cells following PEG2 exposure. In addition, investigations shall aim to determine whether PGE_2 effects TCR signalling. Calcium signalling shall be analysed using widefield microscopy of Fura-2 dyed CL4 following 24hr PGE_2 exposure.

In order to determine the effect of PGE2 on the function of CTLs both kill and proliferation assays shall be completed using CTLs cultured with/without the presence of 1um PGE2 from priming.

1.9.2 Develop novel tumour model

Much of the work in our lab has focused on the interactions of CL4 CD8 T cells with the Renca cells. To determine whether there are common or unique processes regarding tumour mediated immune suppression associated with the Renca and other tumour cell types we wish to develop new BALB/c models of tumour derived from other tissue origins. CT26 cell line consists of fibroblasts derived from a colorectal carcinoma(180). 4T1 cell line is an epithelial line originating from a mammary carcinoma; and is highly metastatic(181). Studies are aimed at characterizing the cell surface molecular phenotype of the 4T1 and CT26 cells by flow cytometry, as this will enable us to ascertain their ability, when pulsed with K^dHA; or later stably transfected with an HA-expressing plasmid, to prime and be recognized by CL4 CD8+ T Cells.

2. Methods and materials

2.1 Media used

Title	Components	Use
Complete Media	RPMI 1640 plus L-glutamine(Gibco), supplemented with 10% FBS(Sigma), 100uM Penicillin Streptomycin(Gibco) and 50uM mecarptoethanol(Gibco).	Cancer cell line culture
T cell Media	RPMI 1640 plus L-glutamine(Gibco), supplemented with 10% FBS(Sigma), 100uM Penicillin streptomycin (Gibco) and 20uM mecarptoethanol(Gibco).	T Cell culture in the absence of IL-2
IL-2 Media	RPMI 1640 plus L-glutamine(Gibco), supplemented with 10% FBS(HyClone), 100uM Penicillin streptomycin(Gibco) and 20uM mecarptoethanol(Gibco), supplemented with 50ul/ml IL-2(NIH/NCI BRB Preclinical Repository)	T Cell culture
Incomplete Media	DMEM(Gibco) supplemented with 10% FBS(HyClone), 100uM Penicillin streptomycin (Gibco) and 100uM Non-essential amino acids(Gibco).	Phoenix cell culture
FACs Buffer	Dulbecco's PBS(Gibco) supplemented with 0.5% BSA(Sigma Aldrich).	Flow Cytometry staining
MACs Buffer	FACs buffer as described above, supplemented with 2mM EDTA 0.02%(Sigma Aldrich).	Magnetic-activated cell sorting
Imaging Buffer	Dulbecco's PBS(Gibco), supplemented with 10% FBS(HyClone) and 100uM MgCL2 and CaCl2.	Live confocal microscopy, wide-field microscopy and Flow cytometry sorting
Chloroquine Saline	Chloroquine diphosphate dissolved in dH2O at 4.1mg/ml(1M)	Retroviral production
Fluorobrite Medium	Fluorobrite DMEM(Gibco), with 10% FBS(Sigma), 100uM Penicillin/Streptomycin(Gibco), and 50uM mecarptoethanol, supplemented with 100uM L-Glutamine	Incucyte imaging-based killing assay

2.2 Mouse breeding and maintenance

2.2.1 Mice

All mice used throughout the project were Thy1.1+/+ BALB/c. Both transgenic CL4+/- mice, expressing monoclonal TCR CD8+ T cells, and CL4 -/- mice were used for immunological studies. All mice were bred and housed at the University of Bristol Animal Services Unit in pathogen free conditions. Any experiments utilising mice were conducted in accordance with U.K Home Office guidelines.

2.2.2 Clone 4 Genotyping

Genotyping was used to determine which bred mice exhibited the CL4 monoclonal CD8+ T Cell phenotype. Blood was extracted through the tail vein of each mouse and diluted using FACS buffer. Samples were centrifuged(1400rpm, 5min) and supernatant discarded. The pellet was resuspended in Ack Lysis buffer(Thermo Fisher Scientific), a solution causing erythrocyte lysis. Cells were washed using FACS buffer and extracellularly antibody stained for CD8b(APC) and Vb8.1(FITC) for 30 minutes before being washed twice to remove any superfluous antibody. Samples were run on a Novocyte 3000 Flow Cytometry analyser. Results were analysed using a gating strategy, found in appendix, identifying the ratio of CD8+ T cells expressing a Vb8.1 TCR allowing positive selection of CL4 mice.

2.3 Tumour Cell Lines

2.3.1 Renca

The adherent Renca cell line stems from a murine renal carcinoma. Throughout the project 2 additional transfected variants of the Renca cell line were used. Renca cells transfected to present

endogenous K^dHA allowing for recognition by CL4 CTLs, deemed Renca HA. In order to maintain a HA presenting population, 100ug/mL geneticin(G418; Sigma-Aldrich) was added to culture media as a selective antibiotic. In addition, Renca wild type cells transfected to emit mCherry red cytoplasmic fluorescence, named Renca mCherry. 250ug/mL hygromycin B(Invitrogen, Carlsbad, USA) was supplemented to the culture media, ensuring all surviving cells emitted mCherry red fluorescence. All Renca derivatives were split at a 1 in 9 ratio every 3 days or once they had reached 60-80% confluence, or 1 in 12 for the weekend.

2.3.2 4T1

The 4T1 cell line was a novel cancer cell line in the Morgan Lab. It is a highly tumorigenic and metastatic, mammary epithelial cancer cell line(181). The cells were subcultured using the same protocol as with Renca Cells however were split at a higher dilution, 1 in 15, every 3 days due to their increased rate of proliferation. It became apparent that any cultures proceeding to grow to 100% confluence resulted in mass cell death. The 4T1 cell line had additionally been transfected to express the luciferase enzyme(Luc), allowing detection during in vivo models(182).

2.3.3 CT26

The CT26 cell line was new to the Morgan Lab. It is a fibroblastic colorectal cancer cell line, reported to metastasis in murine models(180). The fibroblastic origin of the cell line enable varied studies and investigations into the collaboration of types of cells within the tumour microenvironment. CT26 cells were split at the same ratios as Renca cells(1:9 and 1:12). Upon reaching 100% confluence CT26 cells were capable of growing upon one and other, inducing the formation of a tight fibroblastic mesh.

2.3.4 Subculture of Tumour cells

The Renca cell line was maintained in either a T25cm^2 or T75cm^2 filter ventilated flasks(Corning, NY, USA). Cultures were stored in a humidified incubator, with 5% CO_2 at 37^{oC}.

When splitting, culture media was discarded, and flask rinsed in 3-5ml of PBS(Gibco) to remove any excess protein. 0.5/1mL Trypsin-EDTA 0.05%(Sigma Aldrich) was added to the flask and returned to the incubator. Every 2-3 minutes the flask was visually examined under a microscope to detect detached cells. Flasks were gently tapped to aid detachment. After all cells had detached the trypsin was quenched using ~3 times the amount complete media and pipetted into a 15ml falcon tube. The suspension was centrifuged at 1200 RPM for 3 minutes. The supernatant was discarded leaving only the pellet of cells, which was then resuspended in fresh media at the required dilution.

2.4 Liquid nitrogen storage

2.4.1 Freeze down

In order to store cancer cell lines for extended periods of time cells were conserved in Liquid nitrogen stores at -196^{oC}. Cells were first detached from flasks using Trypsin-EDTA 0.05%(Sigma Aldrich). Once detached cells were counted and resuspended at 1-2x10^6 cells/mL in Freeze buffer. Freeze buffer was formed of 90% FBS(Gibco) and 10% DMSO. Once resuspended the solution was quickly aliquoted into Liquid Nitrogen flasks. The aliquots were placed in a 'Mr frosty' followed by a -80^{oC} freezer for 24hrs, up to 3 months. For extended periods of time the aliquots were transported into liquid nitrogen stores.

2.4.2 Thaw

Cells were thawed rapidly in hand/37^{oC} waterbath, gentled pipetted into 9ml complete medium using a pasteur pipette and centrifuged at 1000rpm for 5 minutes. Supernatant was discarded, cells resuspended in 5ml complete medium and plated into a T25 cell culture flask before being incubated at 37^{oC}.

2.5 Cell Counting

2.5.1 Haemocytometer

10uL of cellular suspension was extracted and mixed with 10ul Trypan-Blue. 10uL of the cellular/trypan-blue solution was extracted, pipetted onto a Haemocytometer and viewed under a microscope. The 4 outer edge square of the haemocytometer were counted and totalled. From the count, the overall total cell count was calculated using the equation:

$$\frac{\text{Total cell count}}{4} \quad \text{x } 2 \text{ x } 10^4 \text{ = cells/mL}$$

2.5.2 FACS sorting of CL4 CTLs

Florescence activated cell sorting was used to ensure correct cell numbers were utilised in sensitive experiments. Cells were resuspended in imaging buffer(10% FBS) and sorted for a variety of factors depending on the cell type and experiment. For kill assays, CTLs were gated on their size, for single cells, and for alive cells using Drax7(Pacific Blue). In addition, CTLs previously transduced to express GFP F-Tractin for imaging studies were sorted on a one long increase in GFP fluorescence to ensure sufficient emission was apparent for actin imaging.

2.6 T Cell Culture

2.6.1 IL-2 supplemented CD8+ T Cell Culture

CL4 +/- mice were killed in a humane manner, by either cervical dislocation or CO2 chamber, in accordance to Schedule 1 protocol and spleen surgically removed. The spleen was dissected, pulverised and washed with RPMI, through a 40um filter, leaving a splenocyte rich solution. The solution was then centrifuged,(1400rpm,5 minutes) and supernatant discarded. The cellular pellet was resuspended in 1mL Ack Lysis solution(Thermo Fisher Scientific) for 3 minutes, whilst continually shaken, to eliminate all erythrocytes. 50mL 0.5% Protein Media was used to quench the lysis buffer and the samples re-centrifuged. Once the supernatant had been discarded, the cellular pellet was resuspended in 5mL T cell media, and counted using a haemocytometer. Any clumps of fat were removed using a pipette tip. Once counted the cells were resuspended at $5x10^6$ cells/mL in T cell media. 1mL of the splenocyte solution was loaded into individual wells of a 24 well plate along with 1ul K^dHA and left overnight, allowing for a mixed lymphocyte reaction. The following morning, all wells were collected into corresponding conditioned falcon tubes and centrifuged(1400rpm, 5 minutes). Supernatant was discarded and cells washed 3 times in RPMI to remove any exogenous KdHA peptide. Cells were resuspended at $2.5x10^6$ cells/mL in IL-2 media and 2mL plated into individual wells of a 24 well plate. IL-2 media was replenished bi-daily over the following two days by gently extracting and discarding 1mL media, replacing it with fresh IL-2 media before re-suspending. If the cells were confluent, each well was split into two by re-suspending the wells before extracting 1mL and moving it to a neighbouring well. 1mL IL-2 media was then added to each corresponding well.

2.6.2 Newly Developed CD8+ T Cell Culture

A CL4-/- mouse were killed in a humane manner, by either cervical dislocation or CO2 chamber, in accordance to Schedule 1 protocol and spleen surgically removed. The CL4-/- spleen was dissected and pulverised through a 40uM filter, washed with RPMI. The resulting suspension was centrifuged and supernatant discarded. 1mL Ack Lysis buffer was used to resuspend the cellular pellet and shaken for 3 minutes, to eliminate all erythrocytes. The solution was quenched using 39mL of 0.5% protein media and centrifuged. Supernatant was discarded and pellet resuspended in 30mL T Cell media. The splenocyte solution was then irradiated for 42 minutes at a distance of 27cm from the gamma irradiation source(Irradiator), exposing splenocytes to 3000 Rads.

Following irradiation, splenocytes were centrifuged, washed once, and resuspended in 1ml T cell media, along with 5ul KdHA peptide. The peptide loaded solution was incubated for 1hr at 37^{oC}. A $CL4^{+/-}$ mouse was killed in a humane manner, by either cervical dislocation or CO2 chamber, in accordance to Schedule 1 protocol, and spleen prepped as previously described. Post Ack Lysis buffer the cells were washed 3 times until being resuspended at $5x10^5$ cells/mL. Once the hour was complete the $CL4^{-/-}$ splenocytes were washed 3 times, counted using a haemocytometer, and resuspended at $5x10^6$ cells/mL. Both solutions were then plated in a 24 well plate, with 1mL of each solution per well, resulting in a 1:10 ratio of $CL4^{+/-}$ to $CL4^{-/-}$ cells respectively. The culture was placed in the humidified incubator 37^{oC} for 96 hours, until needed.

2.7 Retroviral transduction of CL4 T cells

2.7.1 Plasmid Mini-prep

Plasmids been previously stored in a bacteria glycerol stock, or as eluted DNA. Competent NEB 5a *E.coli* were heat shocked inducing the uptake of pre-existing eluted DNA. The competent E.coli was thawed on ice. Once thawed ~1-5ul eluted DNA was added to the cells, and kept on ice for 20-30 minutes. The DNA and cell solution was placed from ice into a 42^{oC} waterbath for 30-60 seconds,

before being returned to ice for an additional 2-3 minutes. 250ul LB broth was added to the solution and sewn onto a supplemented agar plate to be grown over night at 37^{oC}. In glycerol stocks, extraction of the plasmid entailed sewing the bacteria onto an Agar plate supplemented with 1% Ampicillin and grown over night. Individual colonies were extracted using a sterile inoculation loop, and used to inoculate 3ml of LB broth, before being placed in a shaking incubator for 16 hours, with the lids ajar. Bacterial overnight culture was centrifuged at 8000rpm for 3 minutes to form a pellet. Supernatant was discarded and pellet resuspended in 250ul Buffer P1(Qiagen) before being transferred into a micro centrifuge tube. 250ul Buffer P2(Qiagen) was added and the tube inverted 4-6 times until the solution becomes clear, as lysis occurs. Within 5 minus 350ul Buffer N3(Qiagen) was added and mixed thoroughly. The solution was centrifuged at 13000 rpm for 10 minutes. 800ul supernatant was then pipetted into a QIAprep 2.0 spin column, centrifuged for 30-60 seconds and flow-through discarded. The wash was repeated using 750ul Buffer PE for 1 minute to discard residual buffer. The spin column was transferred into a sterile 1.5ml eppendorf tube. 50ul Buffer EB or autoclaved deionised water was added to the center of the QIAprep 2.0 spin column, let to rest for 1 minute and centrifuged for 1 minute at 13000rpm to elute the DNA. DNA samples were quantified using a Nanodrop spectrophotometer(Thermo Fisher Scientific, Karlsruhe, Germany)

2.7.2 Plasmid Digest

To ensure the plasmid acquired contained the correct gene a plasmid digest was completed. 3ul of eluted plasmid DNA was combined with the restriction enzymes: NDE1 and STU, and incubated for 1hr at 37c. A control of 3ul of the undigested of DNA was diluted in ddH20. Agarose powder and ddH2O was mixed and the resulting solution gently warmed until all agarose had fully dissolved. Once clear, the solution was poured into a gel template and wells created using a pronged comb. When solid, the gel was slid onto an electrophoresis unit and submerged in 1% TAE(Tris base, acetic acid and EDTA). All samples were mixed with loading dye and pipetted into their corresponding wells, at the negative end of unit. 1Kb molecular weight ladders were loaded either

sides of the samples to enable the calculation of their sizes. The current was turned on and gel run for 1 hour. Upon completion the gel was removed from the unit and photographed using a Transilluminator for imaging and analysis.

2.7.3 Phoenix Cells

Phoenix cells are a mammalian retroviral producing cell line used to generate retroviruses for transfection. Phoenix cells are cultured in 3ml incomplete media. 1ml of 0.02% of EDTA(Thermo Fisher Scientific) was used to detach phoenix cells from them plate. Once detached, cells were extracted and centrifuged at 1200rpm for 3 minutes and supernatant discarded. The cells were resuspended in 6ml fresh Incomplete media, before being split and re-plated in a cell culture dish(Corning).

2.7.4 Retroviral production

In order to transfect Phoenix cells, 25ul Chloroquine was added drop-wise to Phoenix cell culture to prevent intracellular lysosome degradation of plasmid DNA. 2X HBS and 2ul NaOH were combined in a 50ml falcon tube(Corning), deemed Tube A. 500ul ddH2O and 62ul 2M CaCl2 was used to dilute 10ul of desired plasmid DNA, before being added drop wise into Tube A, whilst air is bubbled through the pre-existing solution. The resulting 1mL of precipitate was added drop-wise, onto the Phoenix cell culture.

2.7.5 Transduction of Clone 4 CTLs

Splenocytes were extracted from the spleen and stimulated with the KdHA peptide for ~18hrs, as previously described. Following stimulation the splenocytes were centrifuged(1400rpm, 5 min) and washed 5 times in RPMI. Before the final centrifuge Phoenix cell media, containing the retrovirus, was drawn from their plates and centrifuged (1200rpm, 3min) in a 15ml falcon tube alongside the

splenocytes. Once complete, the splenocyte media was discarded whilst 6ml of the Phoenix cell media was extracted leaving both pellets undisturbed at the base of the falcon tube. The pellet of splenocytes was resuspended in the retrovirus containing media and plated into 3 wells of a 6 well plate. This was then placed in a centrifuge(2hr, 1200rpm, 37c) initiating spinduction. Following spinduction the media was extracted, ensuring to not disturb the cells which were then resuspended in IL-2 media and cultured for 72hrs.

2.8 Live cell imaging of CTLs

2.8.1 K^dHA pulsing

Renca mCherry cells were used as APCs for imaging experiments. Renca mCherry cells were detached from their flask using Trypsin-EDTA(0.05%)(Thermo Fisher Scientific). Once detached cells, were quenched using complete media and extracted into a 15mL falcon tube. Samples were centrifuged(1200rpm, 3min), and resuspended in 1mL of complete media. The cells were counted and resuspended to make $1x10^6$ cells/mL. 2ul/mL K^dHA peptide was then added into the suspension and incubated for 60 minutes. Following incubation the suspension was centrifuged and washed twice using complete media. once washed the cells were resuspended at $5x10^5$ cells in 225uL.

2.8.2 Live cell microscopy

Renca-HA cells are counted and resuspended at $1x10^6$ cells/mL. 1ml of cells is extracted and 2uL K^dHA peptide added for 60mins. $6x10^4$ conditioned CD8 T cells are sorted into an eppendorf containing 2X conditioned imaging buffer. Once peptide loading is complete, the Renca-HA cells are centrifuged and washed 3 times. Once washed, the $1x10^6$ cells are resuspended in 450uL imaging buffer. CL4 CTLs were centrifuged in a microfuge. Imaging buffer was discarded, and cells

resuspended in 15uL of 2X conditioned imaging buffer. 50uL of imaging buffer was added into one well of a 384 well glass bottom plate, followed by 5uL of CL4 CTLs. Once all settled, the glass bottomed plate was placed onto of an oiled confocal or widefield microscope, and focused ensuring no air bubbles formed between the plate and lens. When in focus, 5uL of Renca-HA cells were added directly onto of the CTLs. As soon as Renca-HA cells fell into range, microscopy images were recorded.

2.8.3 Fura Staining

1uL Fura-2 dye was added to the CTLs, samples vortexed and incubated, 30 minutes RT. Peptide pulsed Renca cells were washed and resuspended to $3x10^5$ cells/mL in imaging buffer. Once incubation was complete CTLs were washed and resuspended in 200uL 2uM PGE_2 imaging buffer. The widefield microscopy images were taken through a 384 well glass bottomed plate. 5uL CTLs were added to wells containing 50uL imaging buffer ensuring no bubbles were formed and placed onto the inverted 40x oil-immersion lense. Once settled 4uL pulsed Renca cells were gently pipetted directly onto on the CTLs and widefield imaging started. DIC, 340nm and 380nm excitation images were taken every 10 seconds for 10 minutes.

2.9 Proliferation assay

2.9.1 CTV Proliferation assay

Each aliquot of CTV dye was thawed at room temperature and dissolved in 20ul DMSO. Resulting solutions were stored at -20^{oC}. CL4 splenocytes were extracted from the spleen and prepared, as previously described. Before priming CL4 cells were washed in PBS and counted. CTV dye was diluted 1uL in 1000uL PBS, per $4x10^6$ CL4 splenocytes. If staining cell lines, the described dilution can be used up to $1x10^6$ cancer cells. Once counted the CL4 splenocytes were resuspended in the

diluted CTV stain and incubated in the dark for 20 minutes at 37^{oC}. Following incubation, the samples were quenched using 5 times, 0.5% protein media, and left in the dark for an additional 15 minutes, RT. Once quenched samples were centrifuged and washed twice in T cell media. Stained CL4 splenocytes were resuspended to 5x10^{5}cells/mL in T cell media and plated using either of the culture methods previously described. If utilising tumour cells to prime, CD8+ T cells were first isolated using MACs before being stained. Following the required culture period, the samples were resuspended and cells Live/Dead stained, in addition to CD8+ stained before being run on a Novocyte Flow Cytometer system.

2.9.2 Irradiation of Tumour cells

A T25(cm^3) flask of tumour cells was detached using 1ml Trypsin-EDTA 0.05%. Once detached the flask was washed using 3mL complete media and suspension collected in a 15mL falcon tube. The cells were centrifuged, and supernatant discarded leaving only the cellular pellet. The pellet was resuspended in 10mL complete media and transferred into a 50mL falcon tube. The flacon tube was then placed 33cm from the irradiation source and exposed for 9780 seconds, resulting in 9200 Rads irradiation. Once complete the samples were centrifuged, supernatant discarded, and cells resuspended for counting. Cells were washed once and resuspended at 1x10^6 cells/mL. 100uL of the resulting suspension was added to individual wells of a 96 well plate along with the addition of MACs separated CL4 CD8+ T cells.

2.9.3 MACs separation of CD8+ T cells

A CL4 spleen was extracted and prepared as previously described. The CL4 suspension was washed twice in MACs buffer(1400rpm, 5min), resuspended in 600uL MACs buffer in addition to 50uL anti-CD8 MACs beads, and incubated for 30 minutes at 4oC. An LS column was placed in a midiMACs magnet, above an open 50mL falcon tube, and wetted using MACs buffer. Once

incubation was complete, cells were washed twice and resuspended in 5ml MACs buffer. The suspension was applied to the LS column(Miltenyi Biotec Ltd). Following the sample, 5mL MACs buffer was used to wash the LS column. Once complete, 5mL MACs buffer was added to the LS column whilst it was removed from the midiMACs magnet. A sterile plunger was used to forcefully push the solution through the column into a 50ml falcon tube, resulting in a CD8+ rich population of cells. Upon collection the CD8+ cells were washed using T cell media, counted and resuspended at $1x10^6$ cells/mL in T cell media.

2.10 Kill Assay

2.10.1 IncuCyte imaging-based kill assay

CTLs were cultured for 96 hours using either the IL-2 supplemented or novel culture method previously described. mCherry-Renca cells were detached from their flask, counted and centrifuged. Supernatant was discarded and the remaining cellular pellet resuspended at $3x10^5$cells/mL. 50uL of mCherry-Renca were plated into individual wells of a glass bottomed 384 well plate and incubated until adherence(~3-4 hours). Once mCherry had adhered the cultured CTLs were harvested and sorted for live cells using fluorescence-activated cell sorting(FACs). $1.5x10^4$ cells were sorted per well of Renca-mCherry cells, to ensure equal numbers of live CTLs would be added to each condition. CTLs were centrifuged on a microfuge and supernatant discarded. CTLs were resuspended to $3x10^5$cells/mL. 50uL of CTLs were added onto of the wells containing adhered mCherry-Renca cells at a 1:1 ratio, before being placed in the Incucyte incubated microscope. The plate was then incubated for 18 hours in an IncuCyte live cell analysis system(Essenbioscience).

2.10.2 Novocyte flow cytometry-based kill assay

CL4 splenocytes were extracted and cultured, using one of the previously described method. Tumour cells were stained with CTV, using the previously described method. Stained tumour cells were counted and resuspended to $1x10^6$ cells/mL in complete media. 1mL cancer cells was transferred into an 15mL falcon tube along with the addition of 2uL K^dHA peptide. All samples were incubated for 60mins at 37^{oC}. Once complete, peptide loaded cancer cells were washed and resuspended to $1x10^6$ cells/mL in complete media. CTLs were harvested, washed and resuspended at $1x10^6$ cells/mL in complete media. 100uL of peptide cancer cells were plated in individual wells of a 96 well plate, in rows of repeats. One row of un-pulsed cancer cells was plated to ensure any effect seen is due to specific recognition of the HA peptide. 100uL CTLs were added to each well, resulting in a 1:1 target to effector ratio. 2 rows of 100ul pulsed cancer cells only were plated, along with 100uL additional complete media, to use as a growth and positive controls. The plate was incubated for 17 hours at 37^{oC}. Upon completion the plate was centrifuged(1400rpm, 5min) and supernatant flicked off. PI was diluted 1:400 in accutase. All wells were resuspended in 150uL PI-accutase solution for 10 minutes, to detach any surviving cells. 1 row of cancer cell only wells was collected into a 1.5mL eppendorf tube and placed in a heat block at 90oC to induce cell death and use as a control. When ready, the positive control was returned to a new sterile row on the plate, and the plate run on the Novocyte flow cytometer system.

2.11 Antibody staining and flow cytometry

2.11.1 Live/Dead Stain

To determine live/dead cells Propidium iodide(PI) and Zombie NIR live/dead fixable dead cell staining kit(Biolegend) were used. PI staining enabled flow cytometry of samples with no need to fix. PI was diluted 1 in 400 in FACs buffer to form a staining solution. Samples were centrifuged and

supernatant discarded, before being resuspended in the PI solution. Resuspended samples were run instantly, and live cells detected through the lack of PI fluorescence.

Each aliquot of Zombie NIR dye was thawed at RT and dissolved in 100ul DMSO, with resulting solutions being stored at -20^{oC}. Samples were washed in PBS, to remove any exogenous protein that may aberrantly interact with the dye. Zmb NIR was diluted in PBS to a 1:100 ratio. Cells were counted using a haemocytometer. Up to 1x10^{6} cells were resuspended per 20uL diluted Zmb NIR solution, and left in the dark at room temperature for 15 minutes. Samples were washed twice using FACs buffer, before either being further stained or fixed using 1% PFA.

2.11.2 Extracellular staining

Extracellular flow cytometry staining was used to identify the expression of cell surface molecules on both tumour cells and CTLs. Live/Dead stained cells were washed twice in FACs buffer to remove any superfluous cell dye. Fc block(Invitrogen) was diluted 1uL in 50uL PBS, and used to prevent aberrant binding of targeted antibodies. 20uL diluted Fc Block was added and samples incubated for 15 minuets at 4^{oC}. Fluorochromes used throughout the project include: Allophycocyanin(APC), Allophycocyanin -Cy7(APC-Cy7), Brilliant Violet 605(BV605), Brilliant Violet 655(BV655), Brilliant Violet 785(BV785), Fluorescein isothiocyanate(FITC), Phycoerythrin(PE), Peridinin Chrlorophyll Protein-Cy5(PerCP-Cy5), and Peridinin Chrlorophyll Protein-Cy7(PerCP-Cy7). Stains were diluted in either FACs buffer or PBS, as shown in Antibody Table. 50ul diluted antibody stain was added, for up to 1x10^{6} cells and incubated at 4^{oC} for 30 minutes before being quenched and washed. Experiments in which cells were stained with multiple fluorochromes also included FMO(Fluorescence minus one) stained samples.

2.11.3 Intracellular Staining

Cells were counted and resuspended to $1x10^6$ cells/mL in PBS. Resulting solutions were aliquoted to 250uL in single FACs tubes per condition tested. A control sample was prepared by adding 250uL pervanadate solution to one sample for 5 minutes at 37^{oC}. All samples were washed in 1mL PBS. Zmb NIR dilution was made 1uL per 100uL PBS, staining samples centrifuged and resuspended in 100uL of the Zmb NIR solution. Samples were incubated in the dark for 15 minutes at RT, before being washed in 1mL FACs buffer. Staining samples were centrifuged,(1400rpm, 5mins) and resuspended in extracellular stains CD8+ and Thy1.1+. Staining occurred for 30 minutes at 4^{oC}. Samples were washed with 1mL FACs buffer and supernatant removed. Cells undergoing intracellular staining were fixed in 100uL phosflow cytofix(BD Bioscience), vortexed and left to incubate for 15 minutes at 4^{oC}. Samples were washed twice, in 1mL FACs buffer, and supernatant discarded. 1mL permeable buffer(BD Biosciences) was added to each sample, vortexed and kept on ice in the dark for 30mins. Cells washed twice in 1mL FACs buffer and supernatant discarded. Intracellular antibody stains were prepared in permeable buffer. Each sample was resuspended in 50uL staining perm buffer solution and incubated at room temperature, 60mins. Samples were washed using 3mL FACs buffer before being fixed in 200uL 1% PFA, and analysed within 1 week.

2.11.4 Statistics

Data was analysed for significance, $p<0.05$, using GraphPad Prism.v7 software. Parametric data sets were compared using either a two tailed Student's T Test or a one-way Anova with Tukey's multiple comparison test. Un-parametric data was analysed using Kruskal-Wallis tests with Dunn's multiple comparison test. When data sets were paired, a RM one-way Anova with Tukey's multiple comparison test or a Friedman test with Dunn's multiple comparison test was used for parametric and un-parametric data sets respectively.

3. Results and Discussion

3.1 PGE2 mediated suppression of T cell signaling

3.1.1 Introduction

Naive T cells are primed, both directly and through cross presentation, within the lymph nodes. Priming occurs through stable interactions between CTLs and cells presenting MHC1/2 bound peptides that are recognised by specific TCR. Specific recognition and interactions occur through the formation of a stable synapse, deemed the immunological synapse(64). The immunological synapse is comprised of an accumulation of TCRs and specific co-signaling receptors, named supramolecular activation clusters(SMACs)(66). Stable SMAC formations induce the intracellular transmission of T cell signaling. It is vital that the immunological synapse is sustained for effective T Cell signaling and therefore for priming, proliferation, and effector function to occur(68).

Previous studies have proposed that one common mechanism of tumour mediated immune suppression is the inhibition of cellular polarisation and maintenance of the immunological synapse(135). An inability to sustain stable immunological synapses has been demonstrated in lymphocytes following tumour infiltration and is believed to be induced through multiple mechanisms within the tumour microenvironment(135). Recent studies have demonstrated the ability of Programmed death receptor-1(PD-1) to induce a polarisation impaired state, in turn disabling the immune synapse, leading to decreased cytolytic capabilities(135). Many soluble molecules have also been identified as potential candidates for inducing a suppressed immunological synapse phenotype, including adenosine and PGE2. Suppression of T Cell signaling can be identified through a collection of phenotypic attributes such as morphology, actin polarisation and subdued intracellular secondary signaling molecules.

3.1.2 Aims

I. To assess the effect of PGE_2 on the stability of the immunological synapse.

II. To determine whether PGE_2 effects intracellular actin dynamics of CTLs.

III. To assess the effect of PGE_2 on TCR signaling.

3.1.3 Does PGE_2 effect the stability of the immunological synapse?

3.1.3.1 Morphology studies

Previous studies have identified the loss of maintenance of a stabile immunological synapse as a potential common immunosuppressive mechanism. Following adoptive transfer, primed CL4 CD8+ T cells were seen to exhibit an inability to maintain a stable immunological synapse, leading to loss of effector function and proliferation upon infiltration into the tumour microenvironment(135). Multiple immunosuppressive molecules are under investigation to determine whether they also induce an unstable immunological synapse as a mechanism of suppression. PGE2 has been suggested to play a role in tumour mediated immunosuppression and has previously been described to inhibit both CTL proliferation and effector function(91).

To determine whether PGE2 has an effect on the maintenance of a stable immune synapse, inducing an immunosuppressive effect, a variety of imaging-based assays were completed. CL4 CD8+ T cells were primed using a mixed lymphocyte reaction and cultured for 96hrs. 1uM PGE2 was added for the final 24 hours to represent potential time spent of CTLs within the tumour microenvironment. Once culture was completed, CL4 CD8+T cells were co-cultured with K^dHA peptide loaded Renca mCherry cells. Interactions between CTLs and the Renca target cells were analysed using spinning disk confocal microscopy.

Morphological attributes were monitored over 15 minutes to determine whether the immunological synapse formed between CTLs and target cells was stable and sustained. CTLs often project lamella towards the target interface, increasing their stability. However, lamella projecting

away from the synapse, named off-site lamella, are indicative of an unstable immunological synapse. The percentage of cells exhibiting off-site lamella formation was recorded and presented in figure 12. Following 24hr culture in 1uM PGE2, 25.8% percent of CL4 CTLs were seen to form off-site lamella throughout the assay. When compared to untreated CL4 CTLs(24.2%), no significant difference was seen in the percentage of cells producing off site lamella. In comparison, both untreated CTLs and CTLs cultured in 1uM PGE2 had a significantly lower percentage of off-site lamella formation than lymphocytes isolated from within the tumour microenvironment(TILs)(50.4%). The results suggests that PGE2 within the tumour microenvironment has little/no involvement in up-regulating off site lamella formation as seen in CTLs following tumour infiltration.

Further phenotypic investigation used differential interference contrast images, from live spinning disk confocal microscopy, to analyse the average interface diameters between CTLs and HA presenting Renca target cells. Shortening of the synapse diameter following initial interaction, was used to identify loss of stable interactions between CD8+ T Cells and their targets. The interface diameters of 50 cell couples were analysed, originating from the initial interaction and spanning 540 seconds, results presented in figure 13. A high level of variation was noted between cell couple interface diameter however no significant decrease in the diameter of the immunological synapse was detected at any point throughout the experiment, suggesting the maintenance of stable synapse interactions. The result therefore suggests that 1um PGE_2 alone is not sufficient to induce the inhibition of the stable formation and maintenance of the immunological synapse, noted in TIL. However, the previously stated investigations only observed external morphological attributes of the cultured CTLs therefore further investigations were carried out to examine the internal cytoskeletal environment.

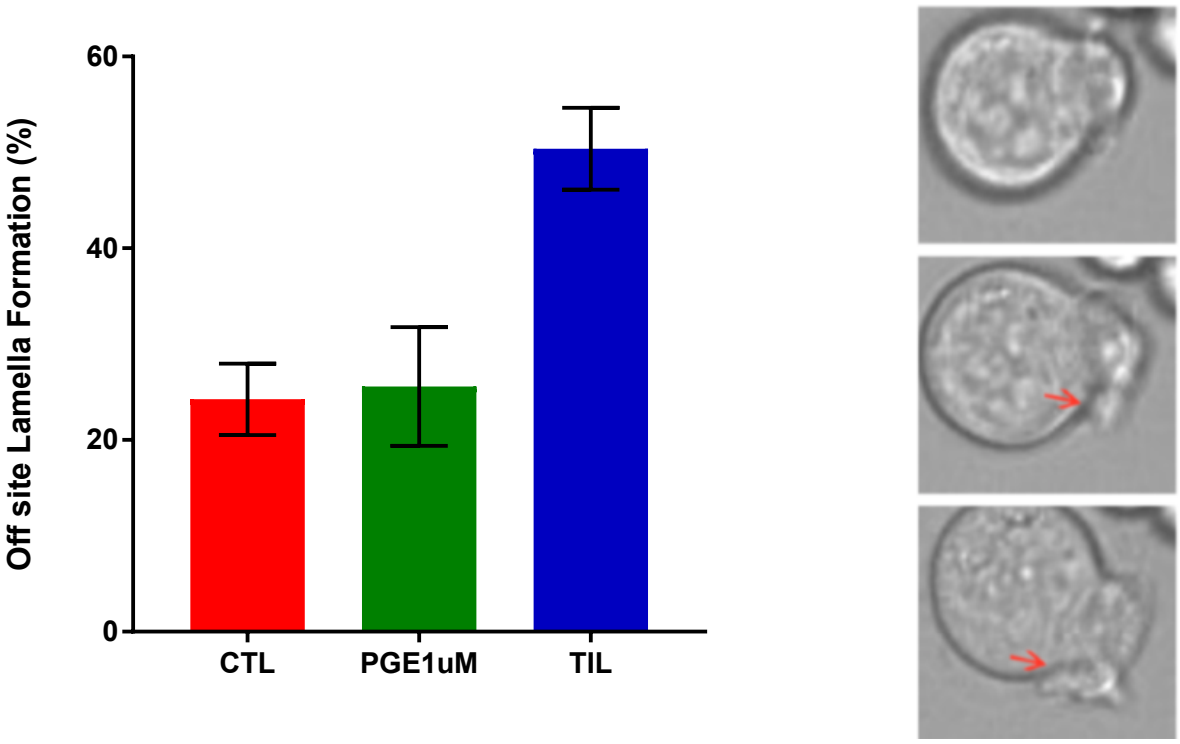


Figure 12: Off site lamella formation

Naive CL4$^{+/-}$ splenocytes were harvested and primed overnight using a mixed lymphocyte reaction with K^{d}HA, as described in methods. Once primed splenocytes were resuspended to 2.5x10^{6} cells/mL in IL-2 media, and 2mL plated into individual wells of a 24 well plate. Resulting cells were cultured for an additional 72 hours. 1uM PGE2 was supplemented for the final 24 hours of CTL culture. When culture was complete, 1x10^{6} Renca mCherry cells were peptide pulsed with 2uL KdHA, for 1hr. Cultured CTLs were harvested, washed and sorted in 1uM PGE_2 imaging buffer, using a BD influx sorter(BD bioscience) for x10^{5} live cells. Resulting CTLs were centrifuged, supernatant gently extracted and resuspended in 200uL 2uM PGE_2 imaging buffer. Once peptide pulsed, Renca cells were washed and resuspended to 3x10^{5} cells/mL in imaging buffer. The microscopy images were taken through a 384 well glass bottomed plate. 5uL CTLs were then added to wells containing 50uL imaging buffer ensuring no bubbles were formed and placed onto the inverted 40x oil-immersion lense. Once settled 4uL pulsed Renca cells were gently pipetted directly onto on the CTLs and imaging started. DIC images were captured every 20 seconds for 15 minutes. Resulting images were analysed for off site lamella formation, using Metamorph computational software. No significant increase in off site lamella formation following 24hr treated with 1uM PGE2 was detected when compared to the untreated CL4 control(n=50). CL4 control and Tumour infiltrating lymphocytes were provided by Dr Rachel Ambler(135) and were recorded over many biological repeats to ensure accurate representative results.

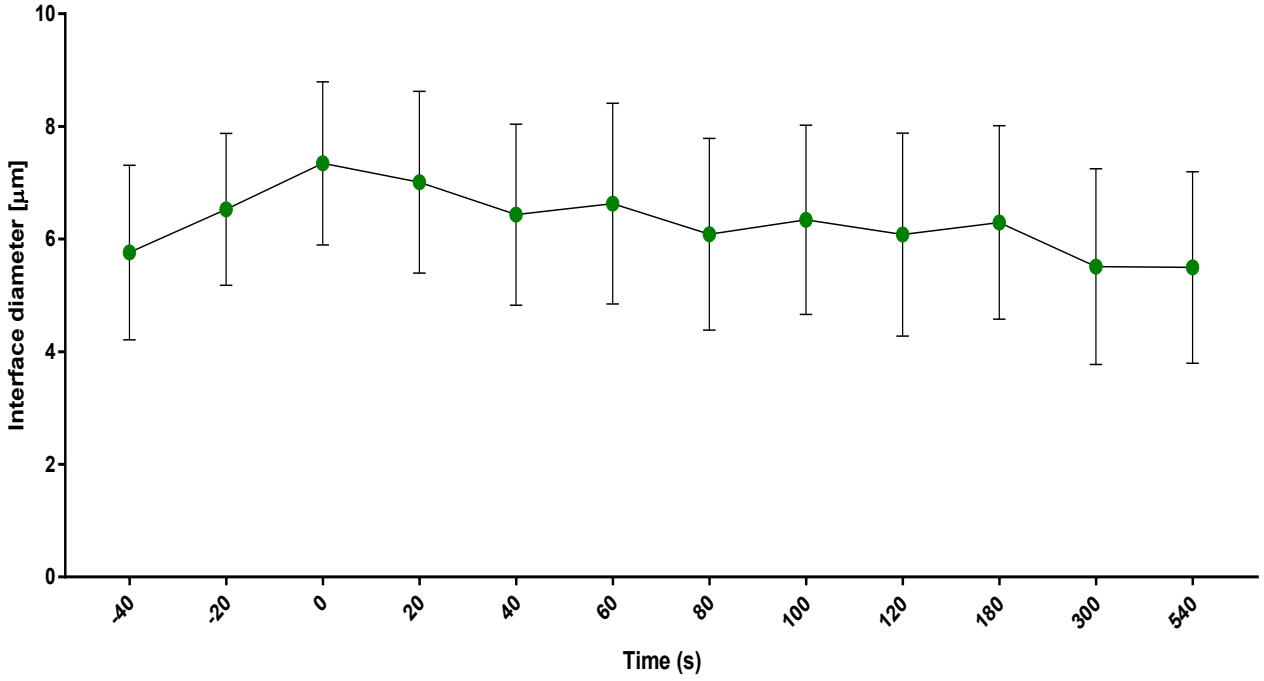


Figure 13: CTL Interface diameter

Naive CL4$^{+/-}$ splenocytes were harvested and primed overnight using a mixed lymphocyte reaction with K^{d}HA, as described in methods. Once primed splenocytes were resuspended to 2.5x10^{6} cells/mL in IL-2 media, and 2mL plated into individual wells of a 24 well plate. Resulting cells were cultured for an additional 72 hours. 1uM PGE2 was supplemented for the final 24 hours of CTL culture. When culture was complete, 1x10^{6} Renca mCherry cells were peptide pulsed with 2uL KdHA, for 1hr. Cultured CTLs were harvested, washed and sorted in 1uM PGE_2 imaging buffer, using a BD influx sorter(BD bioscience) for x10^{5} live cells. Resulting CTLs were centrifuged, supernatant gently extracted and resuspended in 200uL 2uM PGE_2 imaging buffer. Once peptide pulsed, Renca cells were washed and resuspended to 3x10^{5} cells/mL in imaging buffer. The microscopy images were taken through a 384 well glass bottomed plate. 5uL CTLs were then added to wells containing 50uL imaging buffer ensuring no bubbles were formed and placed onto the inverted 40x oil-immersion lense. Once settled 4uL pulsed Renca cells were gently pipetted directly onto on the CTLs and imaging started. DIC images were captured every 20 seconds for 15 minutes. Resulting images were analysed, and interface diameters measured, using Metamorph computational software. No significant changes in the interface diameter were detected across the duration of the assay.(n=50)

3.1.3.2 Does PGE2 effect the actin dynamics of the immunological synapse?

In order for cytotoxic T cells to complete their effector function against target cells, including cancer cells, they must produce a stable immunological synapse, following recognition. The immunological synapse comprises a tight, cell coupling, interface that enables the release of cytolytic granules, containing both perforin and granzymes, in a specific targeted manner, preventing adjacent cell damage(183). The immunological synapse is maintained through the formation and maintenance of a peripheral actin ring at the interface, stabilising the interaction(184). Studies have identified impaired CTL synapse maintenance as an induced suppressed phenotype, following CTL infiltration into the tumour microenvironment(135). Suppression was partially rendered through the loss of a sustained actin presence at the immunological synapse, resulting in the destabilisation of peripheral actin ring formations, and therefore synapse function(135). PD-1was identified to have a key role in inducing the polarisation impaired state seen in tumour infiltrating lymphocytes(135). PD-1 is but one mechanism unregulated within the tumour microenvironment to aid immune evasion. PGE2 is commonly unregulated within multiple cancer types and is believed to utilised as an immuno-suppressive mechanism by cancer cells(186).

In order to determine whether or not PGE2 contributes towards inducing the impaired maintenance of CTL immunological synapses, the pattern of interface actin accumulation of CTLs following 1uM PGE2 treatment were investigated. F-tractin-GFP transduced CL4 CD8+ T cells were co-cultured with KdHA presenting Renca mCherry cells as targets. The culture was examined using epifluorescent live spinning disk confocal microscopy over 15 minutes, with GFP Fluorescence and DIC images taken every 20 seconds. CTL:Target cell conjugates were detected and actin dynamics analysed, 40 seconds before and 480 seconds after initial synapse formation. Resulting data was compared against both CTL controls and TIL data(collected by Dr Rachel Ambler).

Total actin accumulation levels depict the ability of CTLs to effectively polarise their intracellular cytoskeleton. 24 hours treatment with1uM PGE2 was not shown to induce any disabling phenotypic changes in CTL interface actin dynamics. As shown in figure 14, the level of

total actin accumulation saw no significant changes throughout the time course, following 1uM PGE2 exposure. TIL data prototypically demonstrates a reduction in actin dynamics, experiencing significantly decreased total actin accumulations at multiple time points across the assay.

Further investigations examined the level of CTL peripheral actin ring formation. Upon first glance the level of peripheral actin ring formation and maintenance in CTLs treated with1uM PGE_2 does appear to be lower across the majority of the assay, placing its time course curve almost midway between CTL and suppressed TILs however when tested the results showed no significant alterations in the overall level and trend in peripheral actin formation and maintenance. At 180 seconds, PGE_2 treated CTLs were seen to have a significantly decreased peripheral actin expression. However, by the following time point, re-sustained levels of peripheral actin ring formation were noted to exceed the untreated control, therefore deeming the overall change non-suppressive. Examination of TIL data clearly demonstrates their inability to form and maintain peripheral actin rings structures, overall suggesting 1uM PGE2 to not have any significant immunosuppressive effect on intracellular actin dynamics.

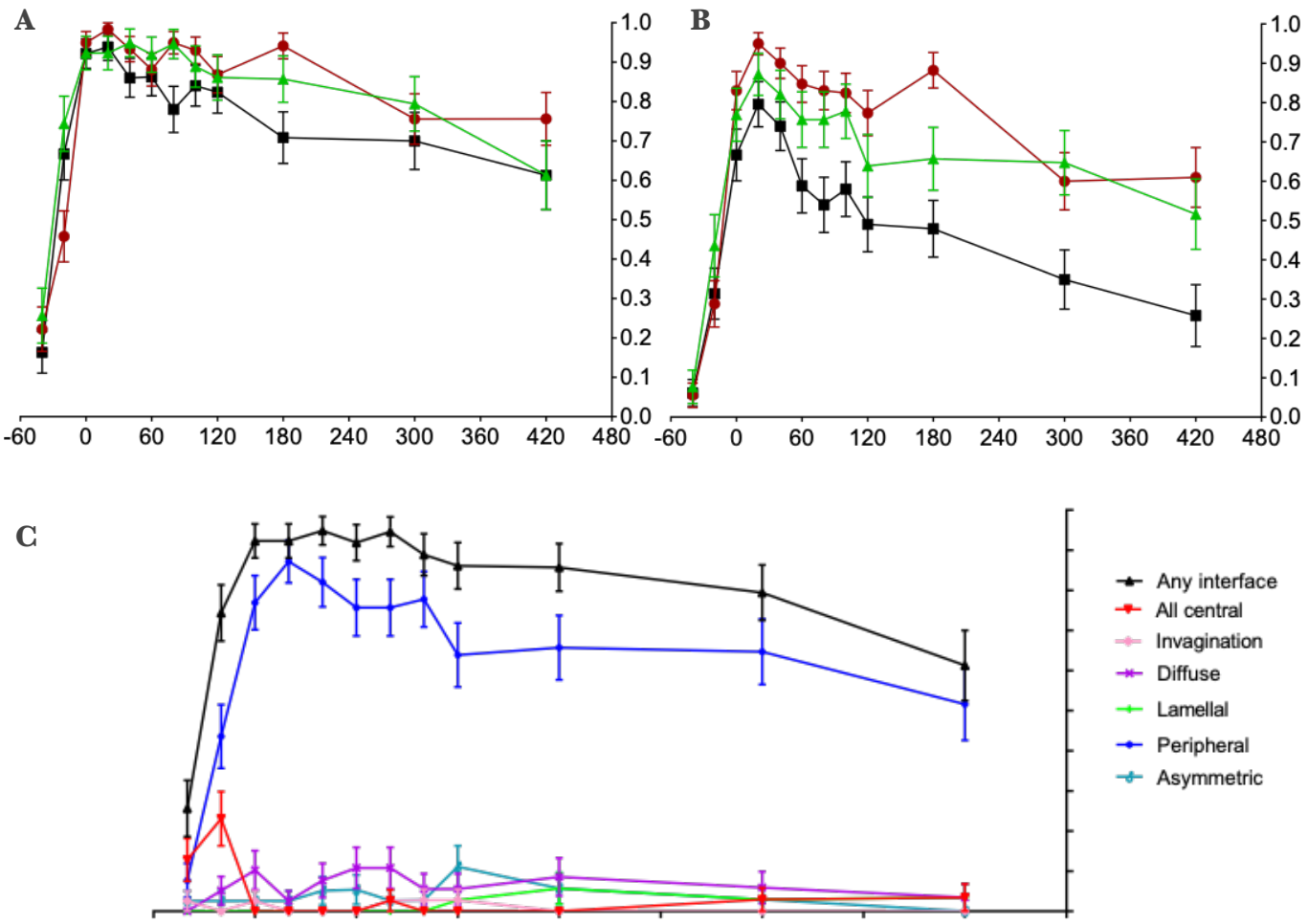


Figure 14: Actin interface patterning.

Naive CL4 splenocytes were harvested and primed overnight using a mixed lymphocyte reaction with K^dHA, as described in methods. 15x10^6 primed splenocytes were harvested, washed and resuspended in 6ml retroviral rich phoenix cell supernatant. 2mL cellular suspension was plated into single wells of 24 well plate, supplemented with 2uL protamine sulphate, and centrifuged for 2hr at 200xg, 32oC. Following spinduction, the phoenix cell media was carefully discarded and each well of cells resuspended in 2mL IL-2 media. Resulting cells were cultured for an additional 72 hours. 1uM PGE2 was supplemented for the final 24 hours of CTL culture. When culture was complete, $1x10^6$ Renca mCherry cells were peptide pulsed with 2uL KdHA, for 1hr. Transduced CTLs were harvested and sorted in imaging buffer, using a BD influx sorter(BD bioscience) for live cells exerting a 1 log increase in GFP emission. Once peptide pulsed, Renca cells were washed and resuspended in imaging buffer. The microscopy images were taken through a 384 well glass bottomed plate. 5uL CTLs were then added to wells containing 50uL imaging buffer ensuring no bubbles were formed and placed onto the inverted 40x oil-immersion lense. Once settled 4uL pulsed Renca cells were gently pipetted directly onto on the CTLs and imaging started. 21 z-stack images were captured every 20 seconds along with a single DIC reference image, for 15 minutes. GFP images were analysed using Metaphorph computational software.(n=48)

A. **Total interface actin accumulation -** 1uM PGE2 treated cells(Green) showed no significant difference in total actin accumulation when compared with untreated CL4(Red) and TILs(Black).

B. **Peripheral actin accumulation -** 1uM PGE2 treated cells(Green) showed no significant difference in peripheral actin maintenance compared with untreated CL4(Red). TILs(Black) demonstrate the destabilisation of peripheral actin formation, indicative of an unstable immune synapse.

C. **Summary actin patterning of 1uM treated CL4 -** High levels of total interface actin accumulation, coupled with efficient maintenance of peripheral actin patterning suggest dully functioning actin polarisation and stable immunological synapse formation.

3.1.2 Does PGE2 effect T cell signaling?

3.1.2.1 Lck signaling

Formation of the immunological synapse is controlled by multiple molecular interactions between the CD8+ T Cell and its target(105). TCR recognition of a specific MHC1 bound peptide enables clustering of both TCRs and co-signaling molecules(106). The formation of these SMACs ultimately leads to the induction of multiple intracellular signaling cascades. When key co-signaling molecules CD8 migrates towards the central SMAC(cSMAC) it brings with it lymphocyte specific protein tyrosine kinase(Lck), bound to its intracellular tail(68). Lck is a crucial kinase that transmits T cell signaling through phosphorylation of multiple downstream signaling complexes. Lck activity is primarily controlled through 2 independent phosphorylations of both Y505 and Y394. Phosphorylation at Y505 is an inhibitory phosphorylation, causing an inactive protein conformation, whereas phosphorylation at Y394 is stimulatory driving kinase activity(67). Migration of Lck towards the peripheral SMAC induces the dephosphorylation of the inhibitory Y505 phosphate group, enabling transphosphorylation and in turn increased Lck activity(69). Phosphorylation has also been reported to occur simultaneously at both sites and is considered active(187). The mechanistic control of Lck activity enables it to be utilised as an indicator of active T Cell signaling.

Therefore, to assess whether intracellular T cell receptor signaling was affected by the presence of PGE2, Lck phosphorylation status of the T cells was investigated following culture in the presence of 1uM PGE_2. Cells were primed and cultured in the absence of IL-2 for 24hrs, before the addition of 1uM PGE2. In addition, a group of cells were treated with TG4-155, an EP2 specific blocker, and further samples treated with both PGE2 and TG4-155. Following a 72hr culture period, the T cells were intracellularly flourescently stained for phosphorylation at Y505, Y394 in addition to total Lck phosphorylation and analysed using flow cytometry. Pervanadate induces phosphorylation of all intracellular proteins and therefore was used as a positive control.

Results, depicted in figure 15, show a decreased percentage(30.1%) of cells exhibiting phosphorylation at both Y505 and Y394(Double positives) following 1uM PGE_2 culture compared to the untreated CTL control(48.9%). In contrast, the results demonstrate an increase in the percentage of cells with solely Y394 phosphorylation(54.2% vs 43.2%) possibly indicating clustering of Lck. However little change in the overall concentration of activate Lck was noted. Nonetheless, an increased percentage of the PGE2 treated cells were seen to lack phosphorylation of both Y505 and Y418(double negative)(15.0%), associated with the inactive conformation Lck, therefore implying a potential decrease T cell signaling efficiency and therefore the possibility of a suppressed phenotype induced via 1uM PGE2. Simultaneous to the potential suppression via PGE2, the results show a substantial increase in the active conformation of sole phosphorylation at Y394 following CTL treatment with TG4-155(73.8%), potentially denoting increased Lck clustering and enhancement of T cell signaling. An increase in T cell signaling following EP2 blockade could imply a role of PGE2 in suppressing SMAC formation and therefore T cell signaling. Yet the overall changes in percentage of phosphorylations were not seen to be significant deeming the test inconclusive.

The lack of significant identification of altered Lck phosphorylation was believed to be due to the inability to isolate cells undergoing cell coupling at the time of analysis. Meanwhile recent studies have developed an imaging-based flow cytometry method that should overcome the issue by enabling isolation of solely coupled cells for analysing in order to detect Lck phosphorylation(189). Therefore instead, further exploration focused on utilising intracellular calcium signaling as evidence of active T cell signaling.

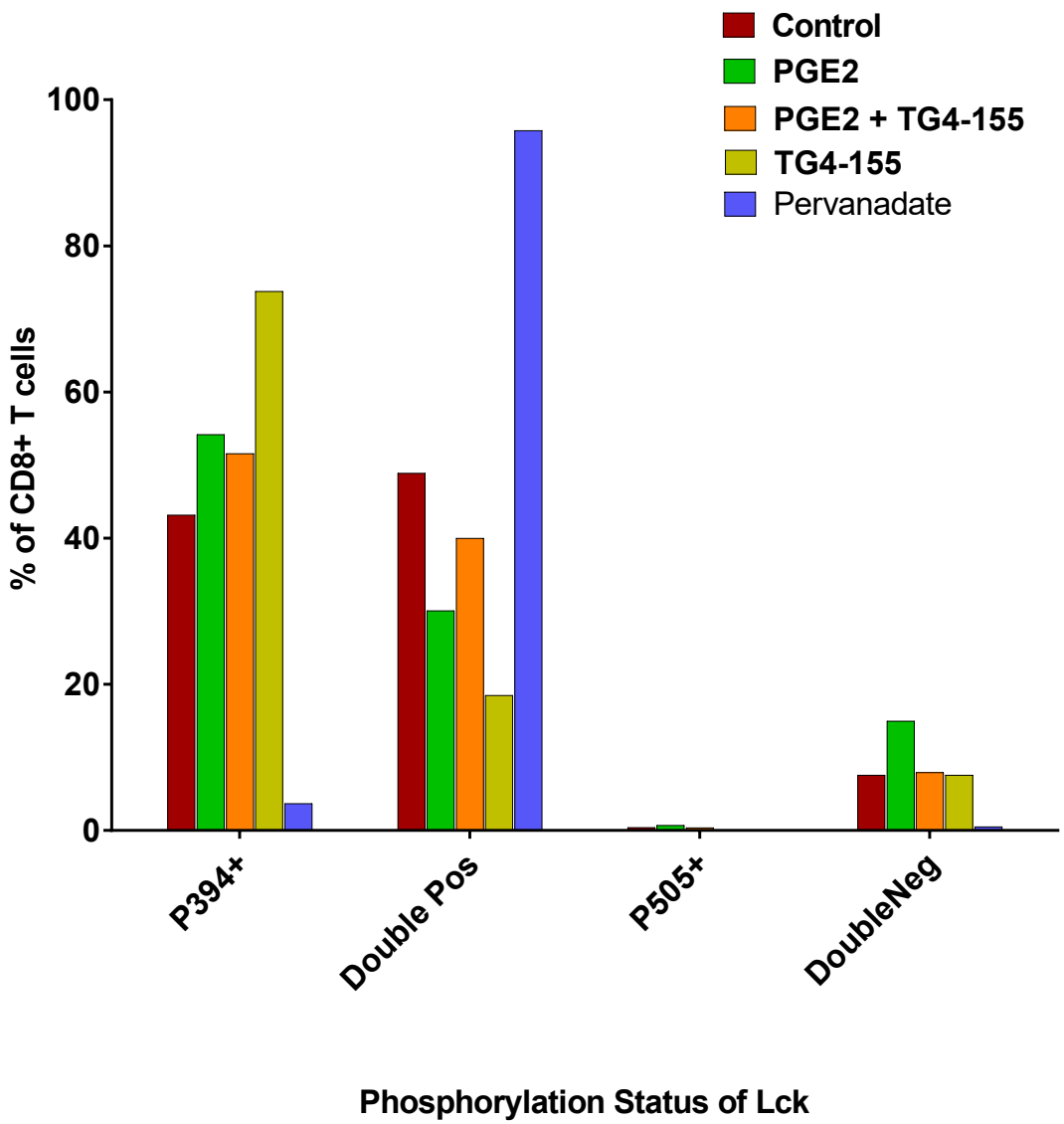


Figure 15: Phosphorylation status of Lck following 1uM PGE2 treatment.

Naive CL4$^{+/-}$ splenocytes were harvested and primed overnight using a mixed lymphocyte reaction with K^{d}HA, as described in methods. Once primed splenocytes were resuspended to 2.5x10^{6} cells/mL in T cell media, and 2mL plated into individual wells of a 24 well plate. Resulting cells were cultured for an additional 48 hours in the presence of 1uM PGE_2. Cells were counted, washed twice and resuspended to 1x10^{6} cells/mL in PBS. 250uL was extracted into 5 FACs tube samples, in addition to necessary USC and FMO controls. One sample was treated with 250uL pervanadate for 5mins at 37^{o}C. All samples were washed in 1mL PBS, and Live/Dead stained using Zmb NIR, using method described. A Zmb only control was fixed in 1% PFA, and remaining samples treated with 50uL Fc Block(diluted 1ul:100uL) for 15 mins, at 4^{o}C. Cells were washed once, and extracellularly stained, along with FMOs, in 50uL diluted antibody for Thy1.1 and CD8b, to identify CL4 CD8+ cells. After 30mins, at 4^{o}C samples were washed in 1mL FACs buffer, fixed in preheated 37^{o}C 100uL phosphlow cytofix, vortexed and incubated for 15mins, 4^{o}C. Samples were washed and resuspended in 1mL perm buffer and kept on ice for 30 mins. Following one additional wash, intracellular staining was completed, as described in methods, using 50uL antibody stain for 60 mins at room temperature. FMOs were made. Intracellular stains targeted: Lck Total, Lck 505 and Lck 418. Anti-Lck 418 antibody was used as a proxy measure as it is only able to bind when Y394 is phosphorylated resulting in active conformation. Samples were washed and fixed in 200uL 1% PFA and run within 1 week. No significant changes in Lck Phosphorylation were noted(n=1).

3.1.2.2 Calcium Signaling

Calcium ions(Ca2+) act as an intracellular secondary messenger, transducing extracellular signals to the internal environment(78). As calcium ions hold a positive charge they are unable to freely move across both the cell surface membrane and the membrane barrier of the endoplasmic reticulum, allowing for their regulation via specific membrane channel proteins, making them extremely useful in signal transduction. Upon T cell:target interaction a path of phosphorylations transmits the initial signal from cell surface receptors to a multitude of the intracellular signaling domain, ultimately inducing the hydrolysis of PIP_2 into DAG and Inositol triphosphate(IP3)(73). IP3 is a signaling molecule that, when produced, induces the release of intracellular Ca2+ ions from endoplasmic reticulum into the cytoplasm(77). The increase in cytoplasmic Ca2+ induces the opening of calcium release-activated cell Ca2+ channels within the cell surface membrane causing a further influx of Ca2+ into the cell. The process of intracellular Ca2+ influx is a key step in the transmission of T cell signaling and therefore allows detection of successful T Cell signaling.

To analyse intracellular calcium signaling CTLs were primed, *via* an MLR, and cultured in IL-2 media for 96hrs. 1uM PGE2 was added for the final 24hrs in attempt to mimic the potential time spent within the tumour microenvironment/spheroid model. Following culture, the CTLs were stained using FURA-2 dye. FURA-2 dye is a fluorescent dye that binds to intracellular calcium that enables detection in changes of calcium levels. The membrane permeant ester nature of FURA-2 allows the dye to freely diffuse into the cytoplasm where it is cleaved by cellular proteases leaving a polarized form of the dye trapped within the cell(190). Once dyed the cells are co-cultured with target Renca cells and examined using UV-widefield microscopy. FURA-2 excites at wavelengths 340 and 380 outputting emissions in a ratio-metric fashion, allowing the calculation of intracellular calcium levels. The average intensity of the emission ratio per cell was recorded, starting 60 seconds before immunological synapse formation and for 8 minutes post, as presented on figure 13.

The graph, figure 16, shows untreated CTLs and those treated with 1uM PGE2 to exert an initial influx of intracellular calcium throughout synapse formation. Untreated CTLs show the peak of their intracellular calcium levels following 1 interval(10 seconds) after initial synapse formation, whereas PGE2 treated CTLs are seen to continually increase for an additional 60 seconds, however the difference seen is not deemed significant implying the occurrence of efficient T cell signaling. In contrast both TILs and spheroid infiltrating lymphocytes(SILs) show little/no significant increase in calcium levels throughout the formation of the immunological synapse spanning the whole 540 second assay, clearly demonstrating evident T cell signalling suppression. Following synapse formation PGE2 treated CTLs show sustained high levels of Ca2+, comparable to that seen in untreated CTLs, showing evidence for continual T Cell signaling and therefore no notable suppression. The results seen, further support that 1uM PGE2 is not sufficient to induce comparable T cell signaling suppression to that seen in TILs/SILs.

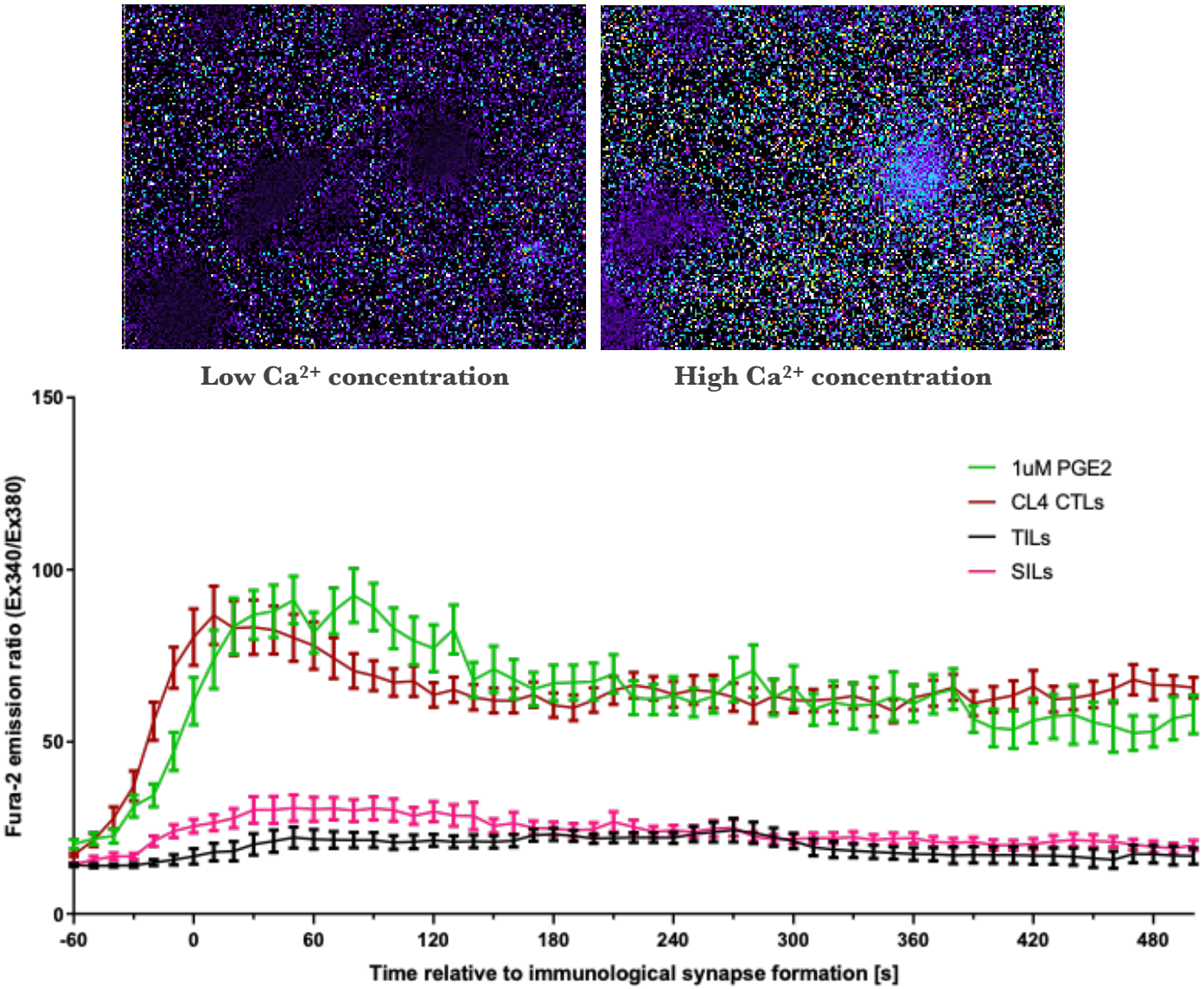


Figure 16: The effects of 1uM PGE2 on intracellular Ca2+ signalling as a measure of T Cell signalling.

Naive CL4$^{+/-}$ splenocytes were harvested and primed overnight using a mixed lymphocyte reaction with K^{d}HA, as described in methods. Once primed splenocytes were resuspended to 2.5x10^{6} cells/mL in IL-2 media, and 2mL plated into individual wells of a 24 well plate. Resulting cells were cultured for an additional 72 hours. 1uM PGE2 was supplemented for the final 24 hours of CTL culture. When culture was complete, 1x10^{6} Renca mCherry cells were peptide pulsed with 2uL K^{d}HA, for 1hr. Cultured CTLs were harvested, washed, resuspended in imagine buffer and sorted into 200ul 2uM PGE_2 imaging buffer, using a BD influx sorter(BD bioscience) for 1.5x10^{5} live cells. Resulting CTLs were centrifuged, supernatant gently extracted and resuspended in 500ul 1uM PGE_2 imaging buffer. 1uL Fura-2 dye was added to the CTLs, and the sample vortexed and incubated for 30 minutes. Once peptide pulsed, Renca cells were washed and resuspended to 3x10^{5} cells/mL in imaging buffer. Once incubation was complete the CTLs were washed and resuspended in 200uL 2uM PGE_2 imaging buffer. The widefield microscopy images were taken through a 384 well glass bottomed plate. 5uL CTLs were added to wells containing 50uL imaging buffer ensuring no bubbles were formed and placed onto the inverted 40x oil-immersion lense. Once settled 4uL pulsed Renca cells were gently pipetted directly onto on the CTLs and widefield imaging started. DIC, 340nm and 380nm excitation images were taken every 10 seconds for 10 minutes. Results show sustained levels of Ca2+ signalling in 1uM PGE2 treated CTLs throughout the duration of the assay.

3.2 PGE2 suppression of cytotoxic T cells

3.2.1 Introduction

CD8+ Cytotoxic T cells play a vital role in tumour elimination through immunosurveillance. One immune evasion method utilised by tumours is the up-regulation and secretion of soluble immunosuppressive molecules, a key example being Prostaglandin E2(PGE2). PGE2 exposure has been demonstrated to induce immunosuppressive phenotypes in a plethora of immune cells.

Studies have demonstrated that 1uM PGE2 is capable of significantly inhibiting activation and proliferation of naive CD8+ T cells(91). The study shows, the 48hr culture of CTLs in 1um PGE2 is sufficient to cause both a decreased percentage of CD8+ T cells reaching the last 2 division, in addition to a 4-fold reduction in the production of IFN-y, identifying PGE2 as a potential immune-suppressive mechanism of direct priming following tumour metastasis(91). This effect was seen to be reversible, with no significant effect seen succeeding secondary culture without consistent 1uM PGE2 supplementation(179).

Inhibition of direct priming of CD8+ T cells is believed to contribute towards tumour growth(Ref). However, in addition CTLs are primed within the lymph node through cross presentation by profession antigen presenting cells(APCs). Antigen bound MHC 1 and 2 complexes are presented on the surface of APCs to naive CTLs along with sufficient co-stimulation through co-signaling molecules CD86/CD80(191). CTLs are activated, via cross presentation, to target a tumour associated antigens and thus infiltrate into the tumour. Following tumour infiltration, activated lymphocytes are seen to exhibit a suppressed phenotype. This is due to a multitude of mechanisms employed by cancer cells that contribute to the tumour immunosuppressive microenvironment, such as soluble immunosuppressive molecules e.g. PGE2.

3.2.2 Aims

I. To assess the effect of PGE_2 on the priming of naive CTLs.

II. To assess the effect of PGE_2 on the cytolytic effect of CTLs.

III. To determine whether the addition of exogenous IL-2 prevents suppression of CTLs by IL-2.

3.2.3 Does PGE_2 suppress priming and proliferation of CTLs?

Previous studies have demonstrated 1uM PGE2 to exhibit a suppressive effect on the priming and proliferation of CD8+ T cells. Naive CD8+ T cells primed using either anti-CD3 + anti-CD28 or anti-CD3 + rICAM-1 in the presence of 1uM PGE2 were seen to exhibit significantly suppressed proliferative and effector function, determined through the decrease in percentage of cells reaching the final two divisions and decreased IFN-y production(91). Further studies confirmed increased levels(1uM) of PGE2 to exhibit suppression in direct priming of CTLs by Renca tumour cells(91). To investigate the effect of PGE2 on proliferation of naive T cells was also seen when priming by cross-presentation via APCs, proliferation assays were completed.

CL4 splenocytes were isolated and stained with Cell Trace Violet(CTV). CTV is a cytoplasmic stain that not only enables detection of specific cells but also depicts the number of cellular divisions. As cells undergo proliferation, they divide into two daughter cells, therefore splitting their intracellular cytoplasmic environment, including CTV, into two. Staining cells with CTV allow for identification of numbers of divisions, represented as loss of CTV fluorescence when analysed using flow cytometry. The stained splenocytes were HA peptide loaded for 17 hours allowing for a mixed lymphocyte reaction(MLR), with or without the addition of varied concentrations of exogenous PGE2(0.1uM/1uM/10uM). MLR induces priming of naive CD8+ T Cells through cross presentation of the HA peptide through endogenous population of antigen presenting cells with the spleen. As only the monoclonal CL4 CD8+ T cell population is specific for the HA peptide, the CTLs undergo extensive priming and proliferation, outgrowing the alternate

splenocytes. Following 17hrs the cells were washed, removing any remaining exogenous HA peptide, and resuspended in splenocyte media supplemented with 50units/uM IL-2(+/- PGE2), and cultured for a further 72hrs. Throughout the culture the media was replenished twice daily to not only ensure sufficient nutrients but also a consistent concentration of PGE2.

Once CL4 CTLs had been identified using the gating strategy, shown in figure 17, the levels of CL4 CTL CTV emission were analysed using FlowJo software, and the percentage of cells reaching a minimum of 4 divisions compared, results presented in figure 18.

In the absence of PGE2 percentage of CTLs to reach at least 5 divisions, was seen to be 59.76%, matching similarly to CTLs treated with 1uM PGE2 at 58.03%. CTLs treated with 0.1uM saw a slight decrease, 53.08%, in the number of CTLs reaching equal levels of proliferation. However, CTLs treated with 10uM PGE2 saw a substantial decrease, with only 41.2% of cells reaching the at least 5 divisions. As not all conditions passed Shapiro-wilk normality test, a Kruskal-Wallis test was used to confirm the significant difference in levels of proliferation. The test confirmed a significant difference in the levels of proliferation following CTL treatment with 10uM PGE2(0.0132, $p<0.05$) when compared to the untreated control. In addition, further significance was also detected when comparing CTLs treated with 1uM and 10uM PGE2(0.0067 $p<0.05$). The results suggest that suppression of CTL proliferation is only seen following treatment with 10uM PGE2.

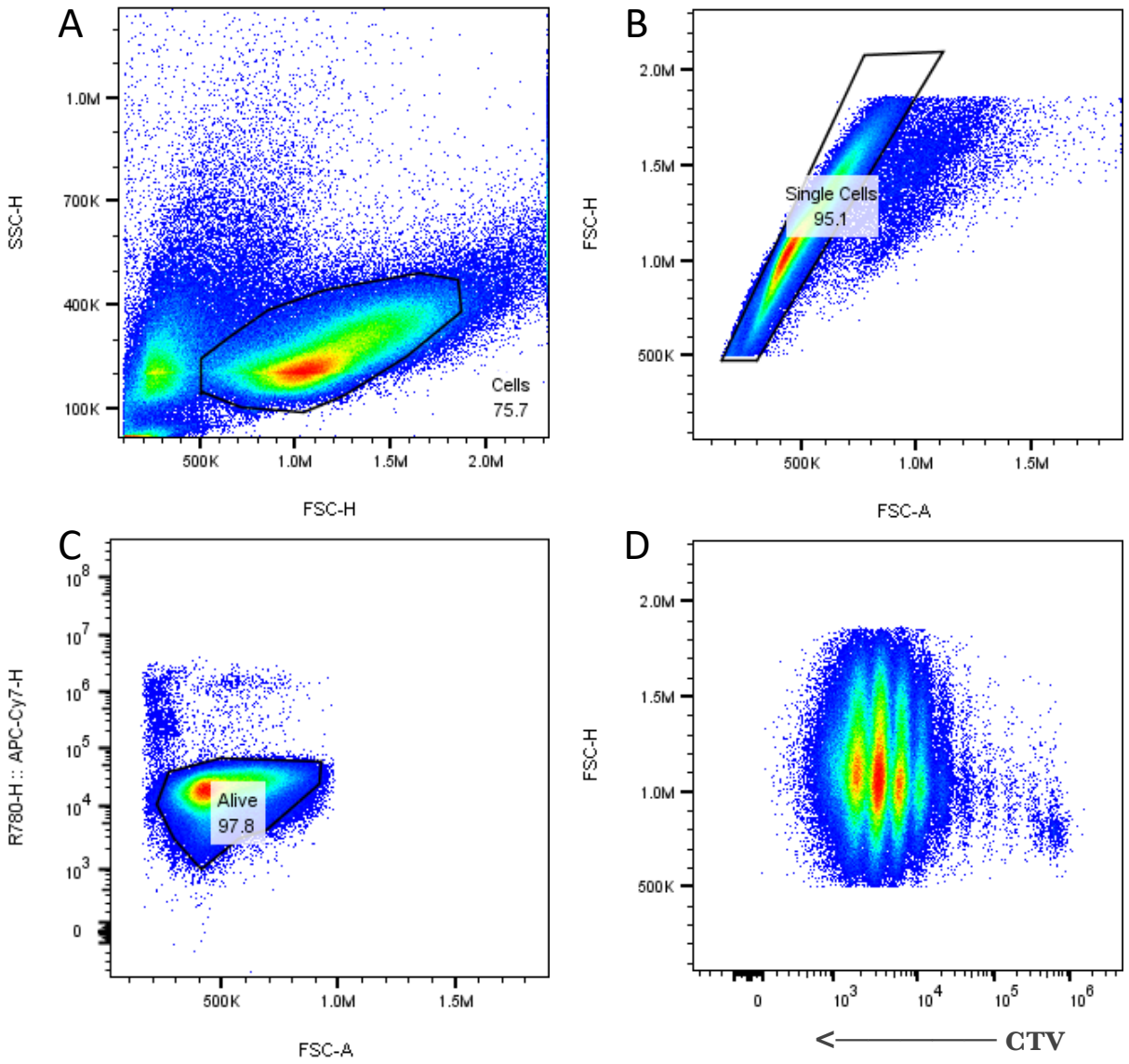


Figure 17: Proliferation assay gating strategy:

Cultured CTV stained CTLs were centrifuged(1400rpm, 5min) and supernatant discarded. Cells were washed twice in PBS. Zmb NIR stain was diluted 1ul in 1000ul PBS. Samples were centrifuged, supernatant discarded, and resuspended in 20uL diluted stain for 15 minutes at room temperature. Live/Dead stained samples were washed twice and resuspended in 200uL FACs buffer.(A) Lymphocytes were determined based on cell size and granularity(FSC-H/SSC-H). (B) Single cells were detected from the lymphocyte population using FSC-H/FSC-A. (C) Zmb NIR Live dead staining allowed detection of alive cells due to the decreased APC-CY7 emission. (D) CTLs were identified through their CTV fluorescence and proliferation determined through decreasing peaks of CTV emission.

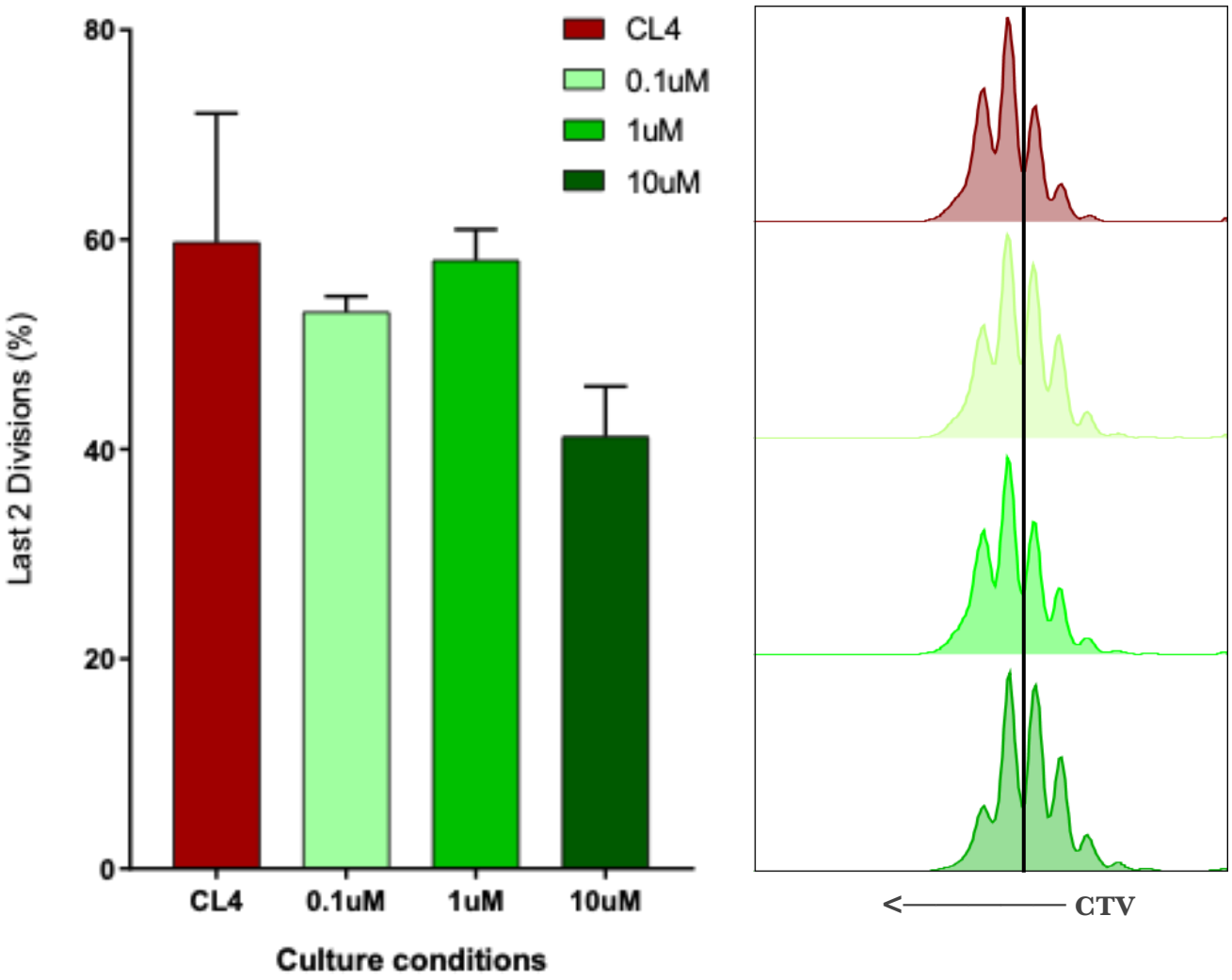


Figure 18: Effect of PGE2 on the proliferation of CTLs.

Naive CL4 splenocytes were harvested, counted and washed twice in PBS. CTV stain was diluted 1uL in 1000uL PBS. 1uL CTV was used per $4x10^6$ cells. Splenocytes were centrifuged, supernatant discarded and resuspended in diluted CTV stain. CTV Staining was completed at 37^{oC} for 15 minutes, followed by a 5x quench in 0.5% protein rich media and an additional 15 minute, dark incubation at room temperature. When complete, cells were washed 3 times in RPMI, re-counted and primed overnight using a mixed lymphocyte reaction with K^dHA, as described in methods. PGE_2 conditions(0uM/0.1uM/1uM/10uM) were added into separate wells at time of priming. $5x10^6$ primed splenocytes per condition were harvested, washed and resuspended in 2ml IL-2 media supplemented with their corresponding PGE_2 conditions. The cellular suspensions were plated into separate wells of a 24 well plate and cultured for 72 hours. Following culture, 1mL cells was extracted from each conditioned well and transferred into individual FACs tubes. Samples were washed twice in PBS and Live/Dead stained using Zmb NIR, as described in methods. Once stained, samples were extracellularly stained for both CD8(APC) and Thy1.1(FITC). Samples were fixed in 1% PFA and run on the Novocyte flow cytometer within 1 week. Analysis utilised a gating strategy, shown in figure 13, to identify alive CL4 CTLs. CTV emission was analysed and percentage of cells in the last two divisions calculated. Kruskall-wallis results show a significant difference($n=2$, $p=0.0036$) between the group means. Individual comparative statistical tests identified a significant reduction in proliferation in CTLs treated with 10uM PGE2($p=0.0132$). No significant difference was noted in cells treated with 0.1uM/1uM PGE_2.

With previous studies demonstrating inhibition of CTL suppression at 1uM PGE2, the results seen were unexpected. Suppressed proliferation was only significantly demonstrated following a 10-fold increase in the concentration of PGE2 than previously reported. Further investigations into the matter, resulted in the belief that the culture method of CTLs was inhibiting the level of suppression seen. The current CTL culture method utilises 50units/ul IL-2 to supplement the culture media. Activated CTLs naturally produce IL-2 to further drive proliferation and differentiation in an autocrine and paracrine fashion. However, the increased levels of IL-2 are considered to be unphysiological and therefore potentially inhibit the level of suppression witnessed in the completed proliferation assays that utilise it in the culture method.

To test this theory additional proliferation assays were completed using the same method on the exception that no exogenous IL-2 was supplemented. In order to determine whether 1uM would be sufficient to induce a suppressed level of proliferation, the cultures were treated with either solely 1uM of PGE2 or PGE2 and TG4-155. TG4-155 is a specific EP2 receptor blockade. By treating cells with both PGE2 and TG4-155 we were able to identify whether it was specifically PGE2 inducing any effects seen. In addition, a group of cells were treated with solely TG4-155 for use as a control. The culture of CTLs in the absence of supplemented Il-2 required the proliferation assay to be completed after 72 hours, in contrast to 96 hours, as this was when the CTLs appeared most prevalent and healthy. Levels of proliferation were compared following FlowJo analysis, using the percentage of cells reaching their final 2 divisions, as seen in figure 19.

Results show a significant difference in proliferation following treatment of 1uM PGE2. 42.43% of cells were seen to reach their final two divisions following treatment with 1uM PGE2, compared to 64.37% in untreated cells. Following the additional treatment of TG4-155 to 1uM pGE2 cultured cells the decrease in proliferation was seen to be partially restored, yet not completely, with 50.6% of cells reaching the final 2 divisions. The only partial restoration post EP2 blockade implies the involvement of additional PGE2 receptors in inducing suppression of CTLs. EP2 and EP4 receptors primarily act in a similar function, both resulting in an increase of

intracellular cAMP. The remaining suppression may, therefore, be a result of EP4 stimulation by PGE2. TG4-155 treatment alone was not seen to affect the level of proliferation(62.4%), ensuring that any effect witnessed was PGE2 dependent.

The results were analysed pairwise, between biological repeats, to account for the high level of variability between murine CTLs. The pattern of proliferation suppression, as seen in figure 15, was consistent across all repeats further suggesting that suppression was being witnessed. A parametric one-way Anova statistical test confirmed the difference seen between groups was significant, with the 1uM PGE2 conditioned cells having a significant reduction in proliferation compared to the alternative conditions.

The results suggest that the up regulation of exogenous PGE_2 reported within tumour microenvironment may be capable of inducing a suppressed phenotype in CTLS, decreasing proliferation of infiltrating lymphocytes and therefore suppressing their population. However, it is evident that the suppressive effect of 1uM PGE2 is only seen in the absence of supplementary IL-2. Comparisons between the two alternative proliferation assays would suggest that IL-2 acts to oppose immunosuppression induced by PGE2, in turn requiring increased levels of suppressive molecules to induce a suppressed proliferative phenotype. Moreover, the counteractive effect of IL-2 on immune suppression has been demonstrated to have an effect on proliferation yet further investigations must be completed to determine whether the effect is also evidence in cytotoxic capabilities.

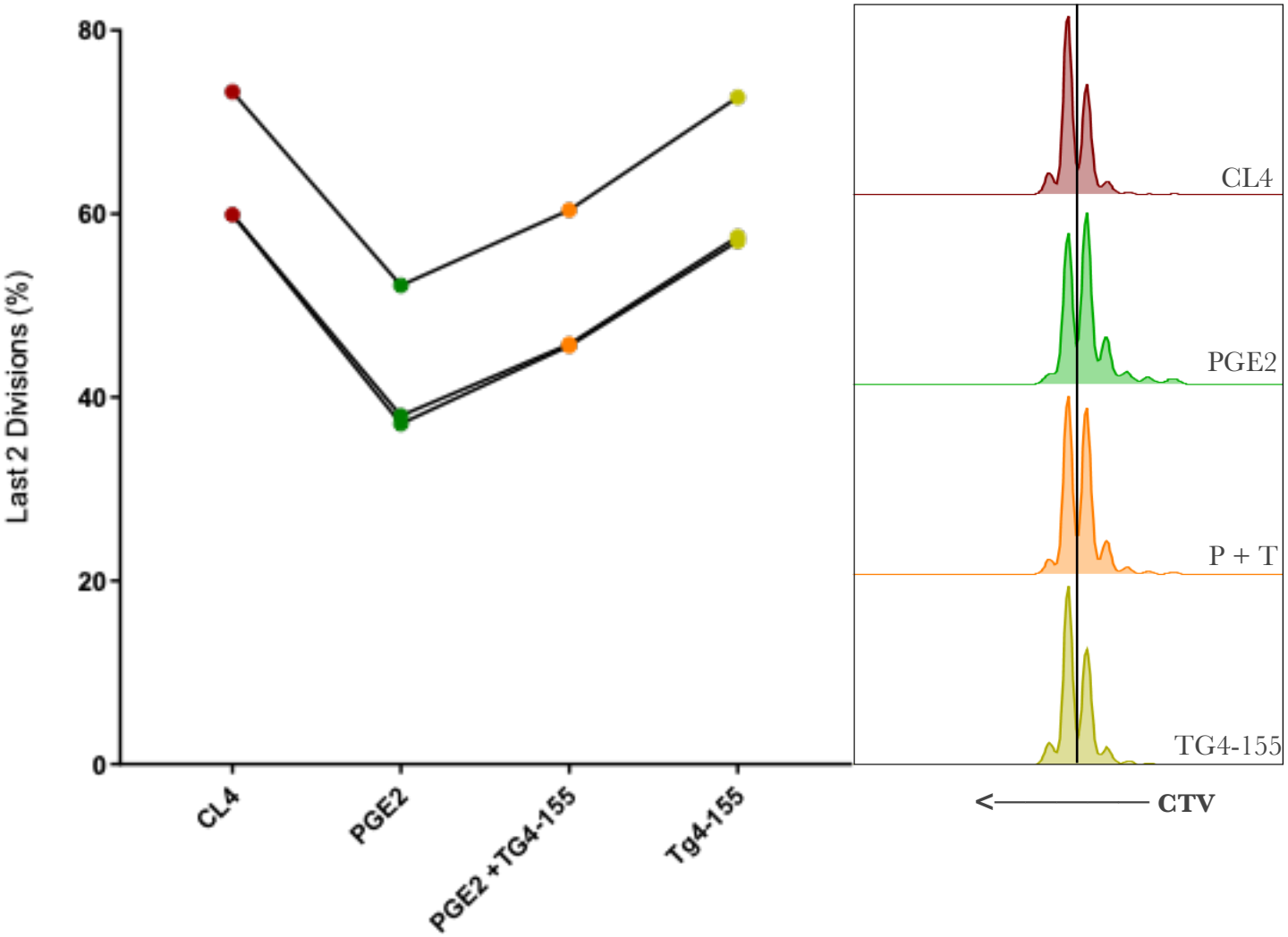


Figure 19: Effect of 1uM PGE2 on the proliferation of CL4 CD8+ T cells when cultured in the absence of IL-2.

Naive CL4 splenocytes were harvested, counted and washed twice in PBS. CTV stain was diluted 1uL in 1000uL PBS. 1uL CTV was used per $4x10^6$ CTLs. Splenocytes were centrifuged, supernatant discarded and resuspended in diluted CTV stain. CTV Staining was completed at 37^{oC} for 15 minutes, followed by a 5x quench in 0.5% protein rich media and an additional 15 minute, dark incubation at room temperature. When complete, cells were washed 3 times in RPMI, re-counted and primed overnight using a mixed lymphocyte reaction with K^dHA, as described in methods. Experimental conditions(1uM PGE_2/1uM PGE_2 + 1uM TG4-155/1uM TG4-155) were added into separate wells at time of priming. $5x10^6$ primed splenocytes per condition were harvested, washed and resuspended in 2ml T cell media with their corresponding conditions. The cellular suspensions were plated into separate wells of a 24 well plate and cultured for 48 hours. Following culture, 1mL cells was extracted from each conditioned well and transferred into individual FACs tubes. Samples were washed twice in PBS and Live/Dead stained using Zmb NIR, as described in methods. Once stained, samples were extracellularly stained for both CD8(APC) and Thy1.1(FITC). Samples were fixed in 1% PFA and run on the Novocyte flow cytometer within 1 week. Analysis utilised a gating strategy, shown in appendix, to identify alive CL4 CTLs. CTV emission was analysed and percentage of cells in the last two divisions calculated. An RM one-way Anova statistical test confirmed a significant difference($n=2$, $p<0.0001$) between the group means. Individual comparisons confirmed a significant reduction($p<0.0001$) in proliferation following 1uM PGE_2 treatment. Suppression was seen to be restored following the additional treatment with TG4-155, confirming the effect was PGE_2. induced.

3.2.4 Does PGE2 suppress cytotoxic function of CTLs?

The key role of CTLs in immunosurveillance is the elimination of transforming/transformed cancer cells, thereby preventing tumour growth(26). CTLs capable of deterring tumour growth recognise cancer specific antigens presented bound to class 1 MHC complexes inducing infiltration into the tumour microenvironment, deeming the CTLs as tumour infiltrating lymphocytes(TILs). Upon recognition CTLs induce cytolysis of cancer cells via three primary mechanism: The release of cytotoxic granules, presentation of the Fas ligand, and the secretion of inflammatory cytokines. The cytolytic action of CTLs acts as a selection pressure within the tumour, leading to the evolution of multiple immunosuppressive mechanisms within the tumour microenvironment.

Many of the investigated immunosuppressive mechanisms, including up regulation of PD-1 and CTLA-4, have demonstrated the induction of impaired cytolytic abilities, presenting a suppressed phenotype of CTLs (192). An additional proposed suppressive mechanism is the up regulation of COX-2 within tumour cells, consequently leading to increased concentrations of PGE2 within the TME (150). 1uM PGE2 has been demonstrated to suppress CTL priming and proliferation via both direct and cross presentation(91). However, the suppressive effects of 1uM PGE2 on proliferation were only witnessed in the absence of IL-2, where10uM was needed for suppression to be seen. To investigate whether PGE2 induced a suppressed cytolytic phenotype in CTLs, a newly developed and optimised flow cytometry-based killing assay was completed.

A flow cytometry-based killing assay was developed utilising Cell Trace Violet(CTV+) cytoplasmic staining to detect target cells and allow quantification of levels of killing. CL4 splenocytes were extracted from a mouse and cultured, in the presence of PGE2 for 72 hours post priming. Untreated CTLs cultured in the absence of IL-2 were used as a control comparison. Renca cells endogenously expressing the HA antigen(Renca HA) were used as target cells. Renca HA were stained using CTV before being peptide pulsed to ensure sufficient levels of HA antigen were presented to induce CTL recognition and cytolysis. Once peptide loaded, target cells and CTLs were co-cultured in a 96 well plate at a 1:1 ratio. Optimisation of the assay,(See appendix)

determined that a 1:1 ratio being the most effective and accurate for detecting a separation between live and dead populations in flow cytometry analysis. The plate was incubated for 17 hours allowing sufficient cytolytic effects to be detected, before being analysed using a NovoCyte flow cytometer system. The resulting data was analysed using the gating strategy seen below, in figure 20.

To determine whether PGE2 had a significant effect on the cytolytic ability of CTLs, the percentage of detected Dead cells were calculated and compared across the culture conditions. Results show an increase in cytolysis of target cells by CTLs when cultured in PGE2 . A Kruskal-Wallis Anova confirmed a significant difference($p=0.0144$) between the means of culture conditions demonstrating PGE2 to have an effect of cytolysis. Further multiple comparisons noted significant difference between the CL4 CTL control and all individual culture conditions containing PGE2(0.1uM <0.0001, 1uM $=0.0286$, 10uM$=0.0022$). However, no difference was noted between the PGE2 culture concentrations thereby determining the effect to be non-dose dependent. The killing assay results suggest PGE2 to aid CTLs in the elimination of tumour cells contrary to pre-existing beliefs.

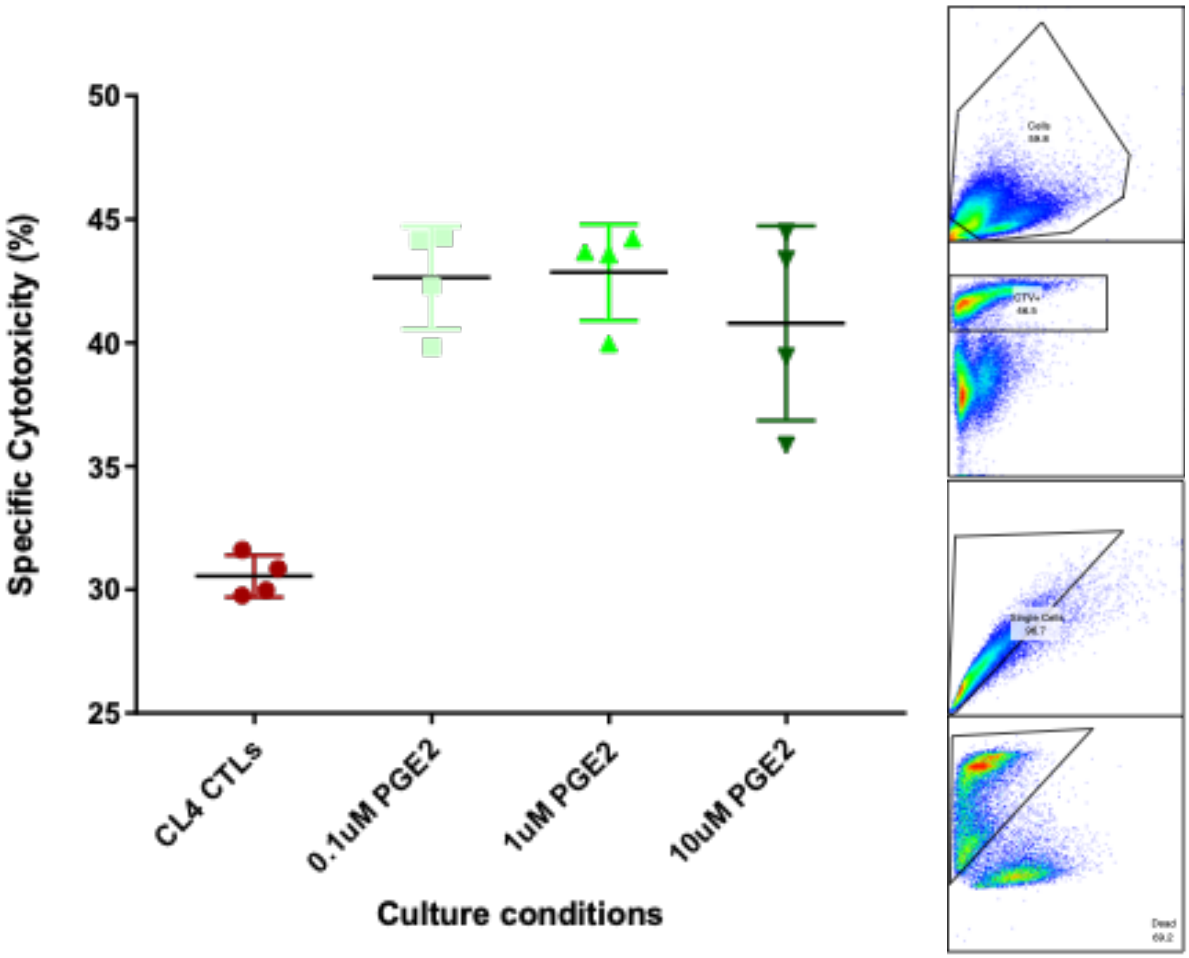


Figure 20: Investigating the effect of PGE2 on CTL specific cytotoxicity

Naive CL4 splenocytes were harvested and primed overnight using a mixed lymphocyte reaction with K^dHA, as described in methods. PGE_2 conditions(0.1uM PGE_2/1uM PGE_2/10uM PGE_2) were added into separate wells at time of priming. $5x10^6$ primed splenocytes per condition were harvested, washed and resuspended in 2ml IL-2 media with their corresponding conditions. The cellular suspensions were plated into separate wells of a 24 well plate and cultured for 96 hours. 60-80% confluent Renca HA cells were detached from a T25 flask using 1mL Trypsin-EDTA. Detached cells were quenched in 4mL complete media, collected into a 15mL falcon tube and centrifuged. Renca HA cells were resuspended in 1mL complete media and counted. Additional complete media was added until $1x10^6$ cells/mL was achieved. 2uL K^dHA was added to the suspension, and incubated for 60 minutes, at 37^{oC}. Once complete cells were centrifuged(1200rpm, 3min), supernatant discarded and the cells washed twice in PBS. CTV stain was diluted 1uL in 1000uL PBS. 1uL CTV was used per $1x10^6$ cancer cells. Renca HA cells were counted, centrifuged, supernatant discarded and resuspended in diluted CTV stain. CTV Staining was completed at 37^{oC} for 15 minutes, followed by a 5x quench in 0.5% protein rich media and an additional 15 minute, dark incubation at room temperature. When complete, cells were washed twice in complete media, re-counted and resuspended to $1x10^6$ cells/mL in complete media. All cultured CTLs were harvested into separate 15mL falcon tubes corresponding to their culture conditions. All CTLs were washed in RPMI, and counted. CTLs were further washed and resuspended to $1x10^6$ cells/mL in complete media. 100ul of both cancer cells and CTLs were co-cultured in repeated wells of a 96 well plate. 4 repeats were plated per condition, in addition to 4 wells of Renca HA only and 4 wells for a positive killed control. The plate was incubated at 37^{oC} for 17 hours. When complete, media from the positive control wells was carefully extracted ensuring not to disturb the adherent cells. 150ul accutase was added to the positive control wells and incubated for 10 minutes at room temperature. Positive control wells were resuspended and collected into a 1.5mL eppendorf. The eppendorf was heated to 90^{oC} for 10 minutes in a heat plate.

The remaining plate was centrifuged at 1400rpm 5 mins, and supernatant discarded. PI was diluted 1uL:400uL in accutase. 150uL PI solution was added to each remaining well, incubated for 10 minutes at room temperature and run within the hour. PI was added to the heated cell accutase at a 1uL:400uL ratio. 150uL kill positive cells were pipetted into a new row of wells in the 96 well plate. All wells were resuspended and run on the Novocyte. Gating strategy seen in figure 16 was used to identify target cells including their live/dead populations. Percentage of dead target cells was determined and specific cytotoxicity calculated using the formula, noted in methods. A Kruskal Wallis anova confirmed significant differences between population means($n=4$, $p=0.0144$). Multiple comparison tests deemed significant differences between CL4 CTL controls and all PGE_2 treated groups(0.1uM, $p<0.0001$ / 1uM, $p<0.0001$ /10uM, $p<0.0003$) . No significant difference was noted between the PGE_2 treated groups.($n=4$)

The newly developed flow cytometry kill assay provides sustained comparable results to investigate the cytolytic ability of CTLs following the addition of PGE2 however one limitation may be the inability to detect a population post apoptotic target cells due to their size and the loss of CTV following cytolysis. To ensure the results being obtained were both accurate and reliable, an additional imaging-based kill assay was completed.

For the IncuCyte imaging-based assay to work, target cells must emit either green or red wavelength cytoplasmic fluorescence to allow detection. Therefore, Renca mCherry cells were used as target cells. Renca mCherry is a Renca cell line that has been transfected with a plasmid to induce cytoplasmic emission of red fluorescence. Renca mCherry were plated into a glass bottomed 384 well plate and incubated until adherence to the plate occurred. Once the mCherry cells had adhered cultured CTLs were harvested and sorted for live cells using fluorescence-activated cell sorting(FACs). Sorting ensured that equal numbers of live CTLs would be co-cultured. The CTLs were able to be sorted solely on live cells as over 90% of the remaining alive cells following 96 hours of culture were CL4 CD8+ cells, demonstrated previously. Once sorted, equal numbers of CTLs were added onto of the wells containing adhered mCherry Renca cells at a 1:1 ratio. The plate was then incubated for 18 hours in a IncuCyte live cell analysis system(Essenbioscience).

In order to quantify the level of cytolytic activity exerted by CTLs following the addition of PGE2. The total surface area of target cells, measured by the area of fluorescence(um2/well), was calculated and averaged across 4 repeat wells. The average at each timepoint was normalised in relation to the first time point to calculate the difference seen over time. Rolling averages were then

calculated at each time point to reduce short term fluctuations and instead emphasise the overall trend. The resulting rolling averages are plotted to allow identification of cytolysis, depicted by the decrease in target surface area, results shown in figure 21.

The results show a clear significant reduction in target cell surface area due to the CL4 CTL cytolysis. However, no significant difference is noted in CL4 treated with 1uM PGE2. Contrary to the previous test, the imaging-based assay concludes that 1uM PGE2 does not have an effect on the cytotoxic ability of CTLs. This is further depicted, in figure 22, by the gradient of the decrease in target cell surface area, as indicative of the rate of cytolysis. No significant effect following the addition of 1uM PGE2 was seen. As the imaging-based kill assay is well established assay and allows the identification of any confounding results through the acquisition of intervallic images, it is believed to be more accurate and reliable than the novel flow cytometry based kill assay, thereby confirming that PGE2 does not induce any significant effect in CTL cytolytic ability.

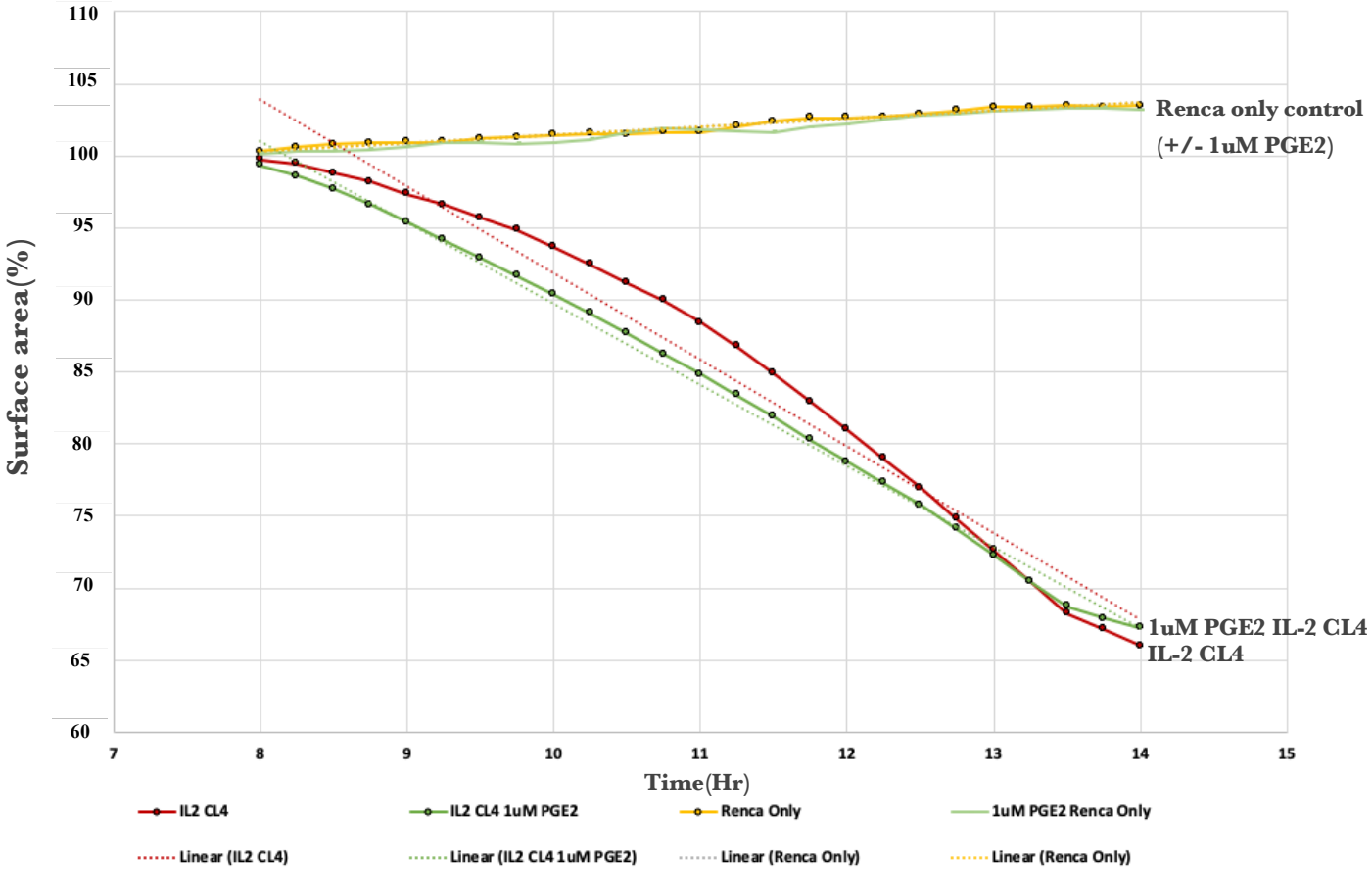


Figure 21: Imaging based kill assay to determine the effect of 1uM PGE2 on CTL cytotoxic abilities

Naive CL4 splenocytes were harvested and primed overnight using a mixed lymphocyte reaction with K^dHA, as described in methods. PGE_2 conditions(0uM PGE_2/1uM PGE_2) were added into separate wells at time of priming. $5x10^6$ primed splenocytes per condition were harvested, washed and resuspended in 2ml IL-2 media with their corresponding conditions. The cellular suspensions were plated into separate wells of a 24 well plate and cultured for 96 hours. 60-80% confluent Renca mCherry cells were detached from a T25 flask using 500uL Trypsin-EDTA. Detached cells were quenched in 4mL complete media, collected into a 15mL falcon tube and centrifuged. Renca mCherry cells were resuspended in 1mL complete media and counted. Additional complete media was added until $1x10^6$ cells/mL was achieved. 2uL K^dHA was added to the suspension, and incubated for 60 minutes, at 37^oC. Cells were centrifuged, supernatant discarded and washed twice using 10mL RPMI. Once washed, Renca mCherry cells were re-counted, and resuspended to $3x10^5$ cells/mL in Fluorobrite media. 50uL Renca mCherry cells were plated in individual wells of a glass bottomed 384 well plate, and incubated until cellular adherence, at 37^oC. 4 repeats per condition were plated. All cultured CTLs were harvested into separate 15mL falcon tubes corresponding to their culture conditions. All CTLs were washed in PBS, and resuspended in 1mL conditioned imaging buffer. 1.5mL eppendorf were prepared containing 200uL 2X conditioned imaging buffer, for each experimental condition.

CTLs were sorted using FACs for 60,000 alive cells per condition into their corresponding eppendorf, taking into account an un-pulsed control group. Sorted CTLs were centrifuged, 4000xg 4minutes, using a microfuge, and supernatant carefully extracted ensuring no disturbance to the cellular pellet. All CTLs were resuspended to $3x10^5$ cells/mL in 2X conditioned Fluorobrite media. 50uL CTLs were added to corresponding target wells containing adhered Renca mCherry, immediately before the plate was placed in the IncuCyte imaging system for 18 hours. Total target cell surface area(um^2/well) was measuring using red fluorescence masking, over the duration of the assay. The mean of 4 repeats was calculated per timepoint for each condition. Results were normalised, and rolling averages produce. Renca only controls(-1uM PGE_2 seen in yellow/+1uM PGE_2 seen in light green) were used to demonstrate cell growth over the duration of the assay. The decrease in target cell surface area was indicative of target cell death via CTL cytolysis. No significant difference was detected between the rate of killing of 1uM PGE2 treated CL4(dark green) and untreated CL4(red).(n=2)

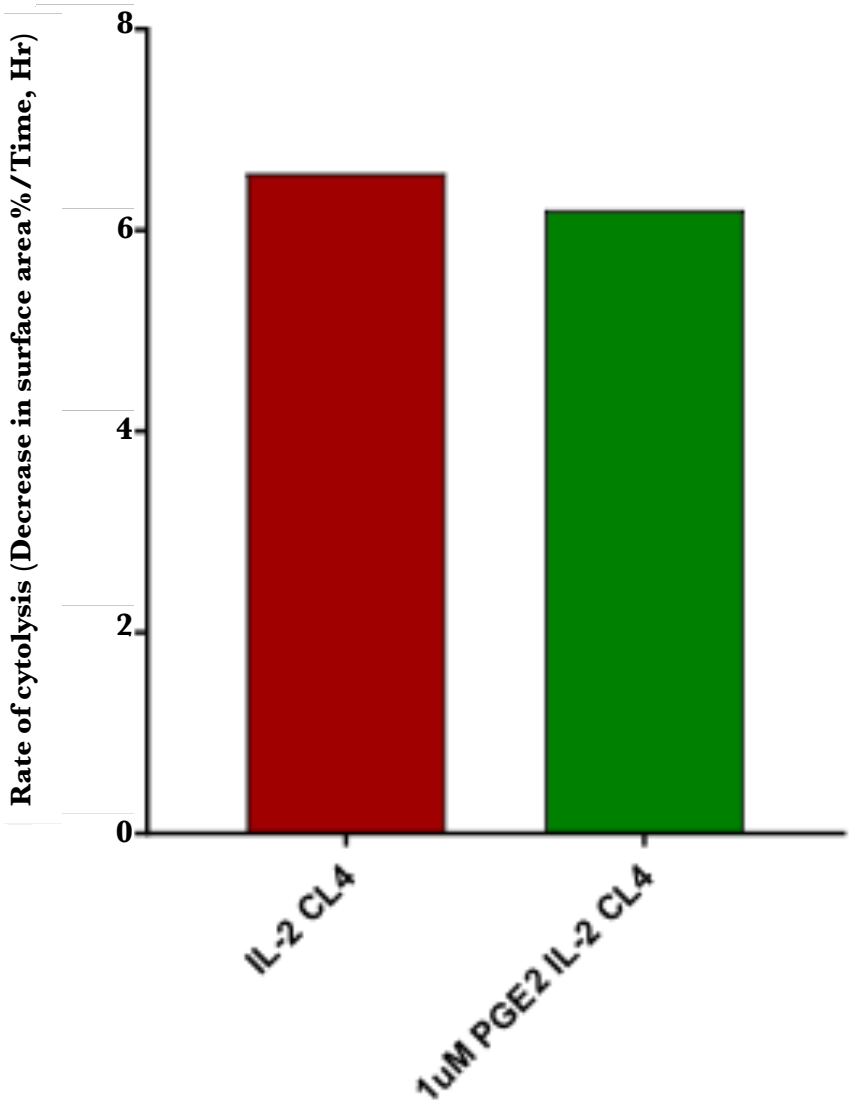


Figure 22: Rate of CTL induced target cytolysis

The gradient of each kill assay time curve was calculated by division of the change in surface area by time. The gradient of the increase in surface area demonstrated by the renca only control, was detracted from the resulting cytolytic gradients to account for the full change in surface are and therefore cytolysis over the time course. The results gradients were plotted on a bar chart to represent the rate of cytolysis. No significant difference in the rate of cytolysis was noted.

3.2.5 Discussion

The contribution of PGE2 on tumour development has been widely investigated. Primarily noted for its up regulation within the tumour microenvironment and its contributing role in Wnt signalling towards the development of colorectal cancer, studies have more recently begun to focus on the effect of PGE2 on the immune system following the increasing interest and development of immunotherapies(186). Historically known as a pro-inflammatory molecule, PGE2 has a multifaceted role in immune-regulation depending on the cell type, maturity and concentration(193). CD8+T cell play a key role in immunosurveillance of cancers and have been shown to exhibit suppressed phenotypes due to multiple up-regulated suppressive mediators within the tumour microenvironment, such as PD-1, CTLA-4 and PGE2(135). Previous studies demonstrated 1uM PGE2 to suppress CD8+ T cell proliferation when primed using classical co-stimulation(91). In addition, 1uM PGE2 was also demonstrated to significantly decrease the level of IFN-y production inferring a decrease in effector function, suggesting a contributing role of PGE2 in tumour mediated immunosuppression(91).

Our study further investigated the immunological effect of PGE2 on CD8+ T Cells, focusing on signalling, proliferation and cytotoxic ability. Live confocal microscopy enabled the evaluation of the stability of the immune synapse. CTLs have been demonstrated to exhibit suppressed phenotypes following infiltration into the tumour microenvironment. Tumour infiltrating lymphocytes were seen to be defective in their ability to polarise their internal cytoskeletal leading to a polarisation in-paired phenotype, in turn suppressing their ability to maintain immunological synapses. The inability to maintain a stable cell couple, depicted by increased off site lamella formation, actin dynamics and an unstable interface, was demonstrated to be induced by the tumour microenvironment. Multiple suppressive mechanisms are known to be utilised by cancer cells to produce a suppressive tumour micro environment. Commonly up-regulated molecules within the tumour microenvironment, such as PD-1 and CTLA-4, were shown to play a key role in the induction of the suppressed phenotype(135). However, when investigated, PGE2 was not seen to

increase the rate of off-site lamella formation. The maintenance in number of off-site lamella formation was coupled with stable synapse formation depicted by maintained interface diameter throughout the 480 second assay. Therefore, the ability to maintain stable immunological synapses following 24hr culture in 1uM PGE2, was clearly witnessed at comparable levels to the untreated CTL control. The findings would suggest that PGE2 is not capable of inducing a polarisation impaired state, implying that PGE2 was not contributing towards the suppressed phenotype seen within the tumour microenvironment.

PD-1 has been proven to play a key role in the induction of defective intracellular actin depolarisation, leading to the inhibition of successful immunological synapse maintenance and therefore the suppression of CTL cytotoxic ability(135). Defective actin polarisation and synapse maintenance has since been suggested as a common path of tumour induced immunosuppression. When investigating whether the up-regulation of PGE2(1uM) seen within the tumour microenvironment played a contributing role in the induction of the defective actin interface seen in TILs, it was demonstrated that 1uM PGE2 was not capable of significantly effecting CTL actin dynamics. Sustained levels of total actin accumulation were seen, confirming no witnessed defects in accent polarisation. Furthermore, no significant changes were noted in the CTLs ability to maintain a peripheral actin ring formation, further supporting the off-site lamella and interface diameter studies, in that stable immunological synapses were maintained. Deeper inspection of the data revealed an in-significant, yet consistent, slight decrease in the level of peripheral actin ring formation potentially suggesting the a contributing role in the decrease in stability was been noted however insignificant. Previous studies have shown 1uM PGE2 to induce CTL suppression in proliferation and effector function, however confounding factors such as IL-2 supplemented culture method used in the actin imaging study may have led to decreased levels of suppression being witnessed, thereby requiring higher concentrations of PGE2 for suppression to be deemed significant. However due to insignificant results, the study cannot conclude that unregulated PGE2

within the tumour microenvironment contributes towards the induction of defective actin polarisation seen in TILs.

Analysis of T Cell signalling, further supported the study by confirming equal levels of TCR signalling were occurring in CTLs following exposure to 1uM PGE2. Investigations into calcium signalling as an indicator of T cell signalling showed tumour infiltrating lymphocytes to have a significantly decreased ability to amount efficient T Cell signalling following interaction with tumour cells. The significant decrease in signalling is clearly shown in tumour infiltrating lymphocytes and explains their defective cytolysis mechanism and suppressed phenotype. CTLs treated with 1uM PGE2 were not seen to exhibit any significant defect in their signalling ability. Overall it was therefore concluded that 1uM PGE2 exerted no suppressive effect on CTL signalling or the formation and maintenance on the immunological synapse. However, previous studies clearly demonstrated CTL proliferation suppression following treatment with 1uM PGE2, therefore further studies were carried out to determine whether the lack of witnessed suppression was due to confounding factors such as the the priming and culture method used within the lab.

Previous studies have demonstrated, suppression of proliferation following exposure to 1uM PGE2 in CTLs primed both via tumour cells directly or plate bound anti-CD3 and anti-CD28/anti-ICAM antibodies(91). However, when investigated using our current culture method no suppression of proliferation was witnessed. Contrary to previous studies, further exploration lead to the discovery that suppression in proliferation was only seen when a 10-fold higher concentration of PGE2(10uM) was used. Furthermore, investigations into the effect of 1uM PGE2 on CTL cytotoxic abilities, demonstrated no significant effects with very high levels of cytolysis still occurring. The requirement of 10uM PGE2 for suppression was unphysiological and unexpected, as previous studies noted 1uM as their highest concentration utilised. Therefore, it was hypothesised that the need for an increased concentration of PGE2 was down to the priming and culture conditions utilised within the lab.

Currently our lab utilises a mixed lymphocyte reaction in which endogenous antigen presenting cells present the HA peptide to naive CD8+ T cells. The cells are then cultured for 96 hours, in which culture media is replenished twice a day. The media used in the culture method is supplemented with very high levels of IL-2(50units/ul) in order to encourage effector CD8+ T cell development and proliferation. However, it is believed that the supplementary IL-2 added to the culture media was also the cause for the lack of witnessed suppression. Further tests into PG2 induced suppression, confirmed this theory showing that when cultured in the absence of IL-2, proliferation of CTLs was significantly inhibited in the presence of 1uM PGE2, suggesting a suppressive role of PGE2. The suppression seen was also partially reverted upon the addition of 1uM TG4-155, a specific EP-2 blocker. Resolution of suppression following the addition of TG4-155, clearly indicates the suppressed effect to be induced by PGE2 specifically, supporting current literature and demonstrating a clear potential immunosuppressive role of PGE2 within the tumour microenvironment. PGE2 is capable of binding 4 individual receptors, EP1-4. Activation of both EP2 and EP4 result in increased levels of intracellular cAMP, capable of inducing suppression(194). As TG4-155 blocks solely EP2, it is believed that the partial resolution is due to the stimulation of the alternative EP receptors, namely EP4, however further investigations must be completed to confirm whether this is correct.

Along with suppressed proliferation, previous studies have established the ability of 1uM PGE2 to decrease IFN-y production, indicating a suppressed effector function, however the cytolytic action was not fully investigated(91). Original kill assays showed no significant effect to the cytolytic ability of CTLs following PGE2 exposure. The lack of effect was also believed to be due to the culture and priming method previously described. IL-2 encourages the formation of IFN-y as well as key cytolytic effector molecules such as perforin and granzyme, therefore the high levels of IL-2 within the culture process is believed to inhibit PGE2 suppression(195). Culturing CTLs using the current culture method without supplementary IL-2 proved troublesome as CTLs would begin to die post 72 hours in culture. Timing of cultures and limited resources made attempting kill assays

using cultures in the absence of IL-2 un-resourceful and inhumane. Therefore, for further investigations into the effect of PGE2 on CTLs, a new culture and priming method was developed to enable more physiological and representative results of the workings within the tumour microenvironment.

Overall the studies would suggest that PGE2 has an immunosuppressive effect on Cytotoxic T cells and therefore could potentially be a contributing mechanism of suppression within the tumour microenvironment. However supplementing IL-2 into CTL culture, as commonly done in adoptive T cell therapy, subverts the suppression seen and dramatically increases the concentration of PGE2 needed for significant suppression to be noted. Additionally, IL-2 notably increases the level of CTL proliferation and effector function, potentially deeming the resulting cells unphysiologically active and effective. Therefore for further,more reliable investigations to occur into the tumour microenvironment, new culture methods should be developed that more accurately represent the internal lymph node environment for analysis and identification of suppressive mechanisms of the tumour microenvironment. In addition, previous experiments using high levels of IL-2 in CTL culture as a control may need to be revisited to determine whether the extent of suppression seen was due to the tested mechanism or due to having enhanced effector CTLs as a compared control.

3.3 Development of novel T cell culture method

3.3.1 Introduction

Current T cell culture method utilises complete cell media supplemented with IL-2(50units/ul) to enhance proliferation and survival of CTLs, in order to sustain growth over the weekend resulting in enough CTLs for the whole lab. A minimum of one CL4 mouse is culled weekly and its spleen harvested. The erythrocytes are eliminated using a lysing agent(Ack Lysis buffer) and the splenocytes isolated. CL4 splenocytes are then primed through a mixed lymphocyte reaction. MLR utilises the endogenous antigen presenting cells within the spleen by loading them with the HA influenza(A/PR/8/H1N1) peptide. The loaded APCs are capable of priming naive CL4 splenocytes through specific TCR signaling. In order to ensure a high yield of CD8+ T cells, following activation the cells are washed and cultured in splenocyte media supplemented with 50units/ul Il-2. Furthermore, culture media is replenished twice daily spanning the weekend to ensure sufficient nutrients for the CTLs, but consequently making CTL culture a demanding process. Using IL-2 in T cell culture provides increased survival, proliferation and effector function of CTLs at 96hrs, and is therefore extremely useful. However, the level of IL-2 utilised is unphysiological and previous results have suggested it holds a role in inhibiting multiple suppressive mechanisms witnessed in the tumour microenvironment, hence its use as an anti-tumour cytokine therapy. The inhibition of T cell suppression has its uses; however, it may also hinder the identification of tumour mediated suppression mechanisms. Therefore, in order to fully investigate the immunological effects of PGE2 a new, more physiological, and overall simplistic CTL culture method was developed.

The new culture method required the use of two mice, one CL4 +/- and one CL4 -/-. The spleen of a CL4 -/- mouse was harvested and the splenocytes extracted. Once extracted the splenocytes were irradiated at 3000 Rads(RX30/55M Irradiator). Following irradiation, the splenocytes were then peptide loaded enabling the endogenous APC population to present the HA

peptide bound to an MHC1. Once peptide loading has begun, a CL4 +/- spleen is harvested and splenocytes extracted as previously described. Succeeding peptide pulsing, both CL4+/- and CL4-/- splenocytes are resresuspended in culture media and plated in a 24 well plate at a 1:100 ratio. The culture can then be left for 96 hours to allow for priming and proliferation, resulting in a simple and effective CTL culture method.

3.3.2 Aims

I. To determine whether the new culture of CTLs enables sufficient proliferation.

II. To optimise and characterise the new culture method.

III. To assess the cytotoxic ability of CTLs following new culture.

IV. To assess the suppressive effect of PGE2 on CTL proliferation in the absence of additional Il-2.

3.3.3 Are CTLs able to effectively proliferate when cultured in the absence of IL-2?

The primary function of CTL culture is to induce and sustain the priming and proliferation of effective CTLs. Therefore, the proliferation of CTLs cultured using the new method was assessed. CTV proliferation assays were completed to detect the percentage of surviving cells, the number of divisions over 96 hours, and the percentage of CTLs reaching the final two divisions. Following 96-hour culture samples were taken from individual wells and analysed using flow cytometry. Live cells were detected through the use of Propidium Iodide(PI). PI is a DNA-binding dye that is actively pumped out by living cells therefore allowing identification of dead/dying cells by the increased PI fluorescence. The percentage of living cells was analysed using FlowJo software and gating strategy and results shown in figure 19.

First lymphocytes were identified through detection of the correct population of cells through forward scatter and side scatter gating. Forward scatter relates to the overall size of the cell, whereas side scatter corresponds to the granularity. As T Cells are both small and typically simplistic with few granules, the main population can originally be identified. Following lymphocyte gating, the cells are gated as single cells. When analysing flow cytometry, it is important to only analyse single cells, as the fluorescence emitted from multiple adhered cells can collaborate inducing flash positive readings. Therefore, single cells are detected on the time and size of cells that pass through the detection laser. Once single cells have been isolated, they can be further analysed to interpret the percentage of remaining live cells. The results, shown in figure 23, demonstrate 87.6% of lymphocytes remaining alive, following 96-hour culture. The post culture results suggest that the culture method is able to sustain enough nutrients whilst providing stimulation over the 96-hour period without the need for media replenishment reducing the workload demand required whilst still producing substantial levels of alive cells.

Using the alive cell gate as previously described, it is possible to examine the proliferation undergone by the CTLs over the culture period. As CTV stained cells divide, their fluorescence emission decreases due to splitting of the cytoplasm, producing a set of uniformly decreasing fluorescence peaks. The individual peaks therefore allow identification of cellular division events.

The results, presented in figure 24, clearly show that the new culture method enables efficient CTL proliferation over 96 hours, with up to 10 cellular divisions seen. Further analysis determined an average of 63.62% of CTLs reach the final 2 divisions. The current culture method reaches an average of 62.02% of CTLS within the final 2 divisions. The comparable results, shown in figure 25, denote the two methods of CTL culture equally proficient in stimulating CTL proliferation over the 96 hours. The maintained level of proliferation, coupled with the high survival rate, of CTLs using the new culture method that does not require the addition of exogenous IL-2, should provide increased opportunities to further investigate tumour mediated immune suppression in a more physiological manner.

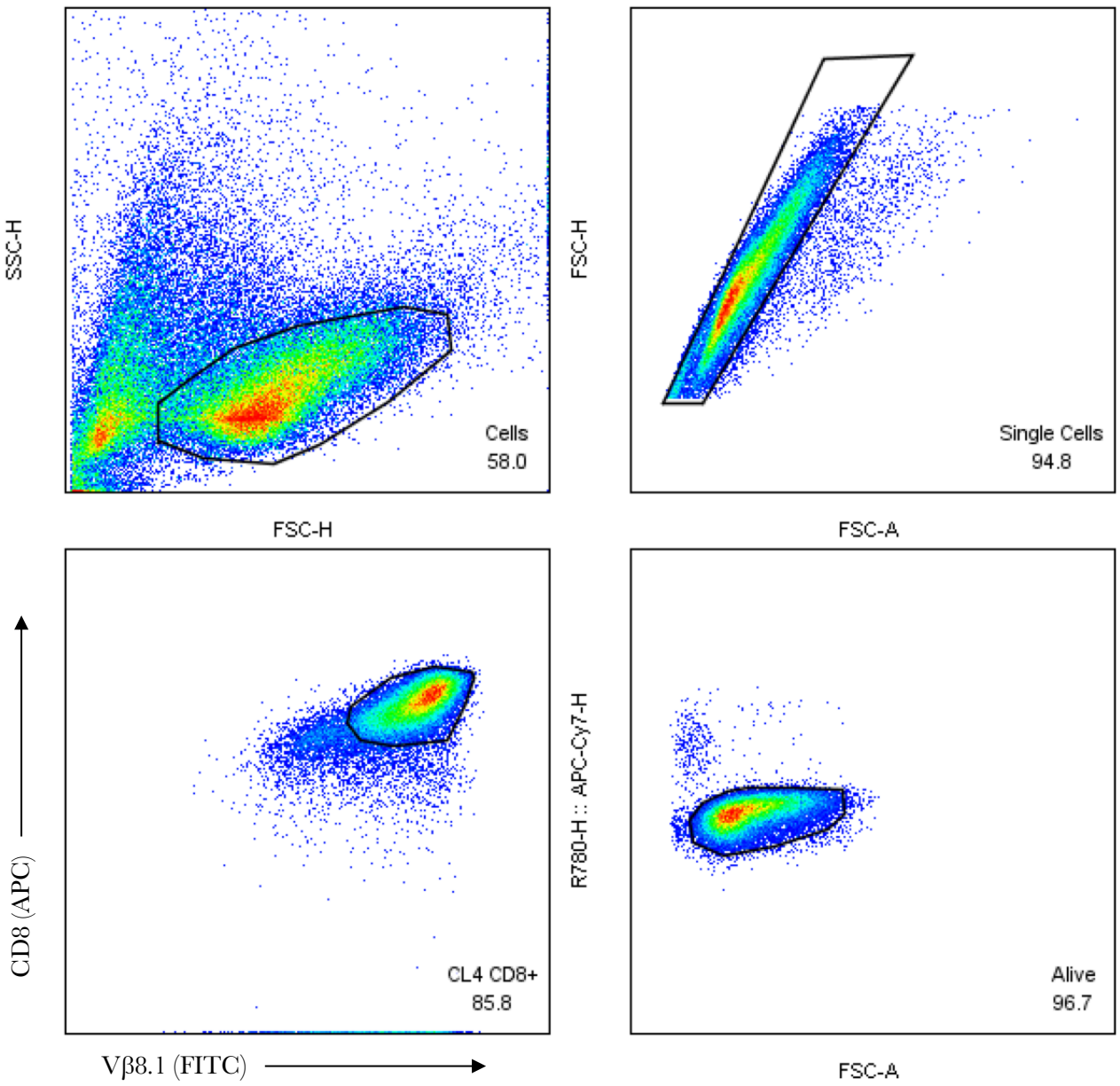


Figure 23: Gating strategy for the identification of CL4 CTLs

CL4$^{-/-}$ splenocytes were harvested and washed twice in RPMI. Cells were resuspended in 30mL T cell media and transferred into a 50mL Falcon tube. Cells were irradiated for 2500 seconds, 30cm away from the source, resulting in 3000 Rads. Once complete, splenocytes were washed twice and resuspended in 1mL T cell media and pulsed with 5uL K^{d}HA for 1hr. Naive CL4$^{+/-}$ splenocytes were harvested, washed twice in RPMI and counted. CL4$^{+/-}$ splenocytes were resuspended at 5x10^{5} cells/mL in T cell media. CL4$^{-/-}$ splenocytes were washed 3 times in RPMI, counted and resuspended to 5x10^{6} cells/mL in T cell media. 1mL of both CL4$^{-/-}$ and CL4 $^{+/-}$ solutions were co-cultured in 8 individual centre wells of a 24 well plate, and left to culture for 96 hours. 250uL cell samples were centrifuged(1400rpm, 5min) and supernatant discarded. Cells were washed twice using FACS buffer. 1uL CD8+ stain(APC) and Vβ8.1 stain(FITC) were diluted 1uL in 100uL FACs buffer. Samples were centrifuged, supernatant discarded, and resuspended in 50uL diluted stain for 30 minutes at 4^{o}C. PI was diluted 1uL:400uL in FACs buffer. Samples were washed twice and resuspended in 200uL PI suspension. Lymphocytes were determined based on cell size and granularity(FSC-H/SSC-H). Single cells were detected from the lymphocyte population using FSC-H/FSC-A. CD8+ cells were isolated, by an increase in APC fluorescence. CD8+ cells were analysed for their Vβ8.1 expression, indicated by increased FITC fluorescence emission. Alive cells were determined by the lack of Zmb NIR/PI emission. 87.6% of CL4 CTLs were identified to still be alive following 96 hour of culture.

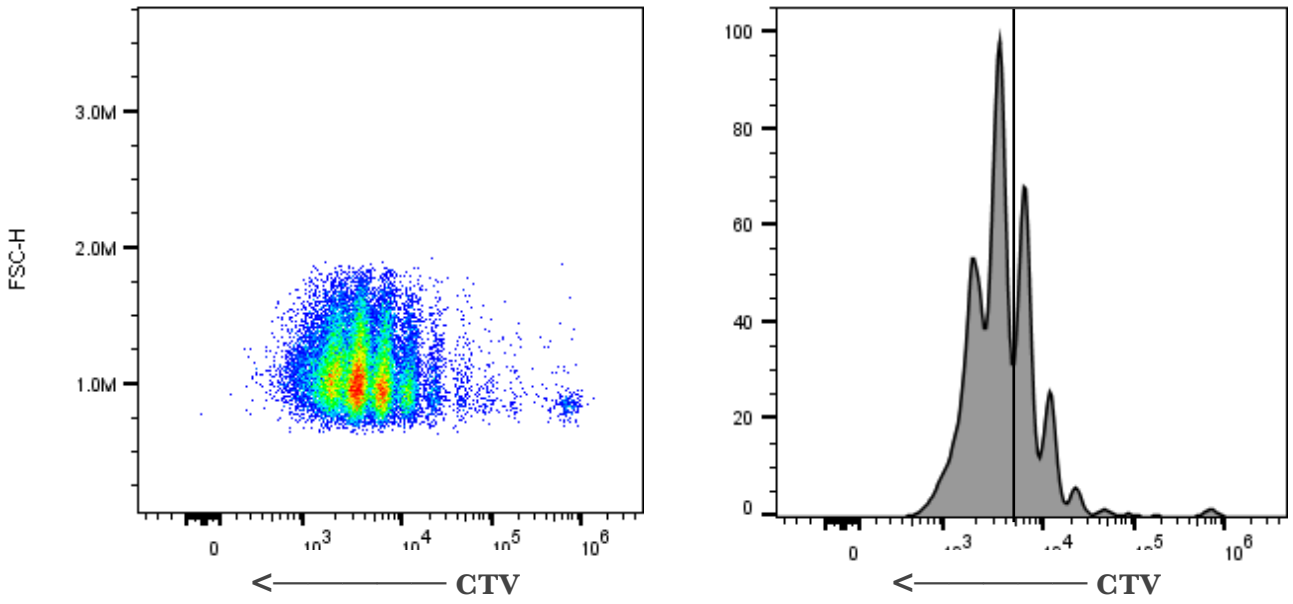


Figure 24: New culture method induces high levels of both CTL survival and proliferation

CL4-/- splenocytes were harvested and washed twice in RPMI. Cells were resuspended in 30mL T cell media and transferred into a 50mL Falcon tube. Cells were irradiated for 2500 seconds, 30cm away from the source, resulting in 3000 Rads. Once complete, splenocytes were washed twice and resuspended in 1mL T cell media and pulsed with 5uL K^dHA for 1hr. Naive CL4 splenocytes were harvested, counted and washed twice in PBS. CTV stain was diluted 1uL in 1000uL PBS. 1uL CTV was used per $4x10^6$ cells. Splenocytes were centrifuged, supernatant discarded and resuspended in diluted CTV stain. CTV Staining was completed at 37^{oC} for 15 minutes, followed by a 5x quench in 0.5% protein rich media and an additional 15 minute, dark incubation at room temperature. When complete, cells were washed 3 times in RPMI, re-counted and resuspended at $5x10^5$ cells/mL in T cell media. CL4-/- splenocytes were washed 3 times in RPMI, counted and resuspended to $5x10^6$ cells/mL in T cell media. 1mL of both cell solutions were co-cultured together in wells of a 24 well plate and cultured for 96 hours. Following culture, 250uL cells was extracted from each well and transferred into FACs tubes. Samples were extracellularly stained for both CD8(APC) and Thy1.1(FITC), using the protocol described in methods. PI was diluted 1uL:400uL FACs buffer. Samples were washed twice in PBS and resuspended in 200ul PI solution, as described in methods. Samples were run on the Novocyte flow cytometer. Analysis utilised a gating strategy, shown in figure 19, to identify alive CL4 CTLs. CTV emission was analysed and percentage of cells in the last two divisions calculated. High levels of proliferation were seen with an average of 62.02% of cells reaching the last two divisions.(n=3)

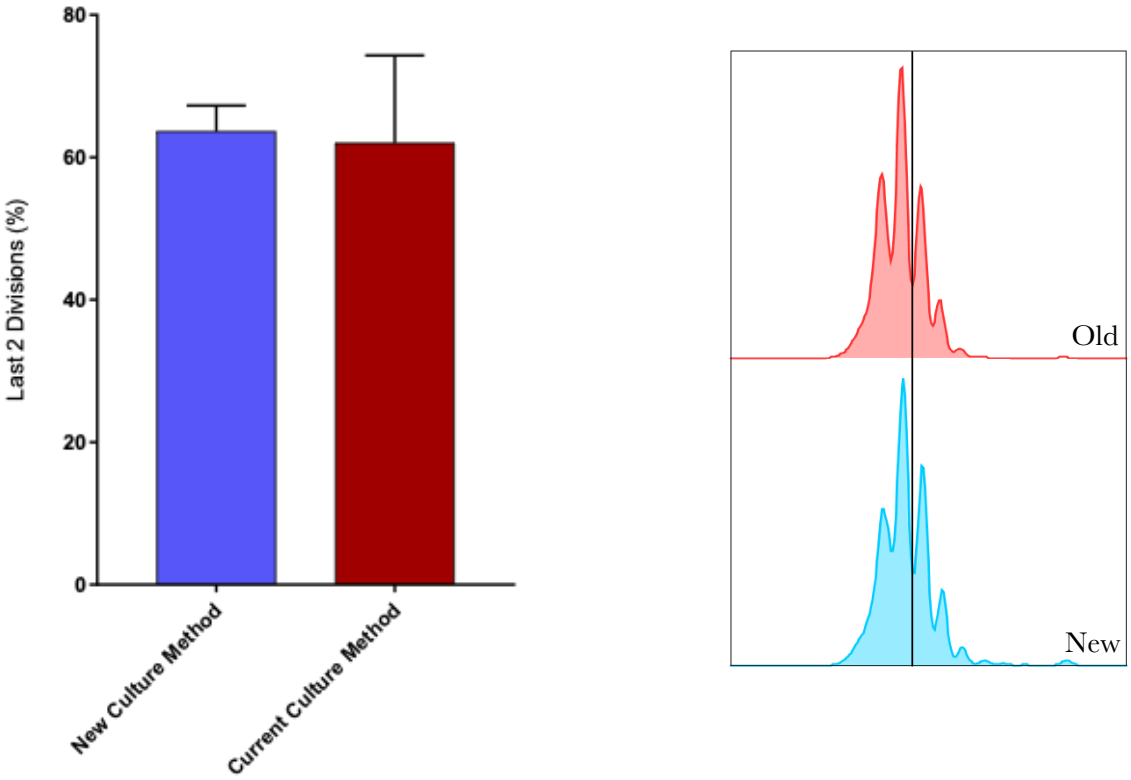


Figure 25: Comparable proliferation of new culture method and IL-2 supplemented CL4$^{-/-}$ splenocytes were harvested and washed twice in RPMI. Cells were resuspended in 30mL T cell media and transferred into a 50mL Falcon tube. Cells were irradiated for 2500 seconds, 30cm away from the source, resulting in 3000 Rads. Once complete, splenocytes were washed twice and resuspended in 1mL T cell media and pulsed with 5uL K^{d}HA for 1hr. Naive CL4$^{+/-}$ splenocytes were harvested, counted and washed twice in PBS. CTV stain was diluted 1uL in 1000uL PBS. 1uL CTV was used per 4x10^{6} cells. Splenocytes were centrifuged, supernatant discarded and resuspended in diluted CTV stain. CTV Staining was completed at 37^{o}C for 15 minutes, followed by a 5x quench in 0.5% protein rich media and an additional 15 minute, dark incubation at room temperature. When complete, cells were washed 3 times in RPMI and re-counted. 4x10^{6} CL4$^{+/-}$ splenocytes were extracted from the total resuspended at 5x10^{5} cells/mL in T cell media. CL4$^{-/-}$ splenocytes were washed 3 times in RPMI, counted and resuspended to 5x10^{6} cells/mL in T cell media. 1mL of both CL4$^{-/-}$ and CL4 $^{+/-}$ solutions were co-cultured in 8 individual centre wells of a 24 well plate, and left to culture for 96 hours. The remaining naive CL4$^{+/-}$ splenocytes were primed overnight using a mixed lymphocyte reaction with K^{d}HA, as described in methods. 5x10^{6} primed splenocytes per condition were harvested, washed and resuspended in 2ml IL-2 media with their corresponding conditions. The cellular suspensions were plated into separate wells of a 24 well plate and cultured for an additional 72 hours. Following culture, 1mL cells was extracted from each well and transferred into FACs tubes. Samples were extracellularly stained for both CD8(APC) and Thy1.1(FITC), using the protocol described in methods. PI was diluted 1uL:400uL FACs buffer. Samples were washed twice in PBS and resuspended in 200ul PI solution, as described in methods. Samples were run on the Novocyte flow cytometer. Analysis utilised a gating strategy, shown in figure 19, to identify alive CL4 CTLs. CTV emission was analysed and percentage of cells in the last two divisions calculated. No significant difference(p=0.7002) in the percentage of CL4$^{+/-}$ CTLs reaching the last two divisions was seen when comparing the newly developed and IL-2 supplemented culture methods.(n=2)

3.3.4 Characterisation of CTLs following new culture.

For the use of the new CTL culture method to become comprehensive it was first optimised and tested to determine the purity of the surviving population. The current culture method used results in an alive population consisting of ~94.6% CL4 lymphocytes. Such a high yield of CL4 CD8+ T cells enables their isolation via Flow cytometry sorting for the alive population. Being able to extract specific numbers of CL4 CD8+ T cells solely through gating on live cells, prevents the need for potentially effector compromising fluorescent staining. Staining for specific molecules such as CD8 or CD3 is possible however the effects of the fluorescent antibodies are poorly understood, as well as the process being time consuming. It is therefore beneficial to result in a high purity of solely CL4 CD8+ T Cells following culture. As both the current and new culture methods induce stimulation through priming with the HA peptide, the only population of primed cells should be CL4 CD8+ T cells as they are the sole group of cells with a TCR specific for HA. However endogenously presented peptides may also be capable of inducing alternative cellular stimulation.

To assess the purity of the population following the new culture method, CTLs were cultured for 96 hours and stained for Thymocyte antigen 1(Thy1.1) and CD8 using fluorescent antibodies. Thy1.1(CD90) is a cell surface protein expressed solely on thymocytes, allowing the immediate detection of the lymphocyte population. Once lymphocytes are isolated, the CD8 stain is capable of distinguishing CTLs specifically through their co-signaling receptor. Using the previously shown gating strategy for live cells, the stained new culture sample was then measured using a NovoCyte flow cytometry system and analysed using FlowJo software, results presented in figure 26.

The population of stained cells was compared against an unstained sample, ensuring that there was a distinct increase in fluorescence and therefore binding of fluorescently labelled antibodies to both Thy1.1 and CD8 molecules. The results demonstrate 86% of the remaining alive cells to be Thy1.1+. Once gating on Thy1.1+ cells, the population is seen to be 93.3% CD8+. The results therefore show the remaining population to have a high yield of CL4 CD8+ T cells therefore allowing the potential for gating solely on live cells in future experiments. In addition, Thy1.1 may

be used to isolate cells in sorting using flow cytometry, as its binding to antibodies has been demonstrated to exhibit no effects to CTL effector function. Sorting on Thy1.1+ cells would therefore allow extraction of a ~93% pure population of CL4 CTLs, for further experimentation.

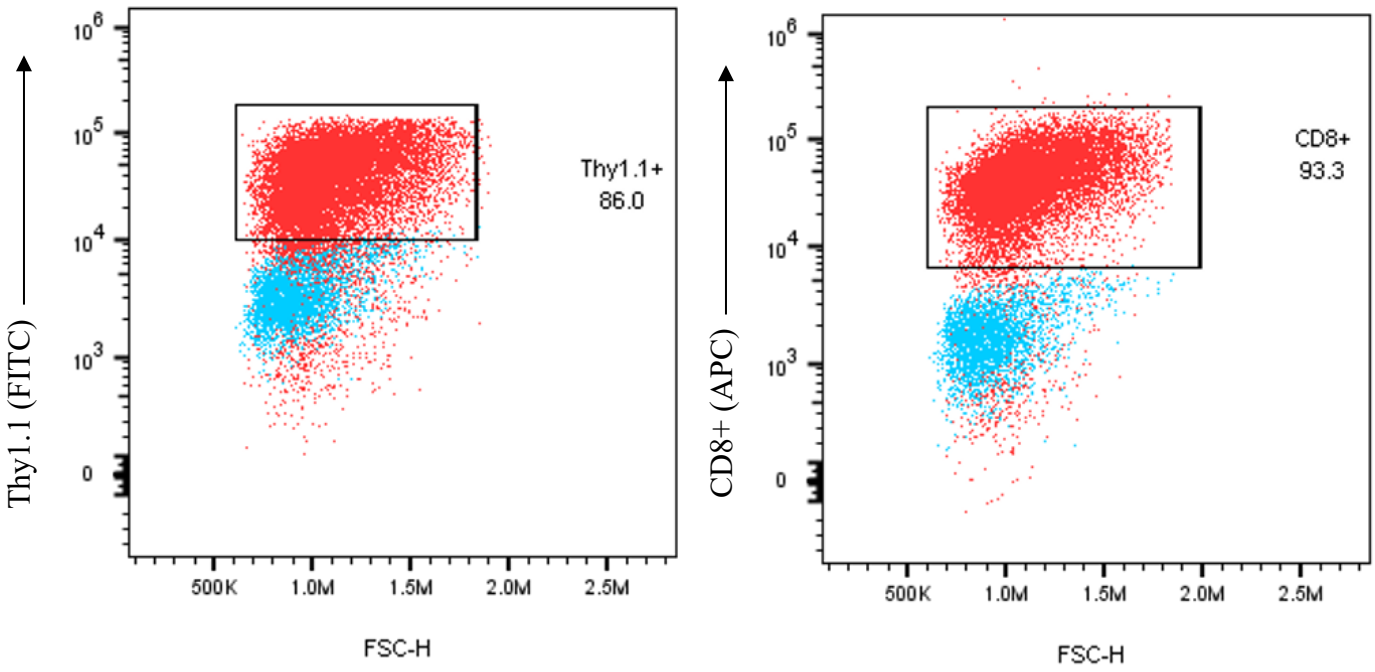


Figure 26: Identification of a high yield of CL4 CD8+ Cells in the surviving population
CL4-/- splenocytes were harvested and washed twice in RPMI. Cells were resuspended in 30mL T cell media and transferred into a 50mL Falcon tube. Cells were irradiated for 2500 seconds, 30cm away from the source, resulting in 3000 Rads. Once complete, splenocytes were washed twice and resuspended in 1mL T cell media and pulsed with 5uL K^dHA for 1hr. Naive CL4 splenocytes were harvested, counted and washed twice in RPMI. When complete, cells were resuspended at $5x10^5$ cells/mL in T cell media. CL4-/- splenocytes were washed 3 times in RPMI, counted and resuspended to $5x10^6$ cells/mL in T cell media. 1mL of both cell solutions were co-cultured together in wells of a 24 well plate and cultured for 96 hours. Following culture, 250uL cells was extracted from each well and transferred into FACs tubes. Samples were live/dead stained using Zmb NIR, using the previously described method. Once stained, cells were washed twice and extracellularly stained for both CD8(APC) and Thy1.1(FITC), using the protocol described in methods. Results show 86% of the remaining alive population of cells to be Thy1.1+. 93.3% of the Thy1.1+ cells, were also seen to be CD8+.(n=3)

Following priming, T cells typically develop into three main categories, T central memory(T_{EM}), T effector memory(T_{CM}), and T effectors cells(196). By identifying extracellular molecular expression patterning, it is capable to determine the majority population phenotype. In order to determine the differentiated type of cells present over the course of the newly developed culture method, a time course characterisation was completed.

The time course entailed 6 days of extracellular staining targeting: CD44, CD62L, CD69, and PD-1. CD44 is a glycoprotein indicative of lymphocyte activation. CD44 can be used to determine activated cells as it is not highly expressed in naive T cells. CD62L is a cell adhesion molecule used by CTLs to home to the lymph nodes and is therefore lost upon naive T cell priming, however it is also characteristic of central memory CTLs and therefore is noted to be unregulated again in differentiated CTLs. When seen in combination with CD44-, CD62L+ indicates naive T cells, however CD62L+ CD44+ cells are recognised as central memory T cells. Upon loss of CD62L(CD62L-) but maintenance of CD44+, cells are deemed effector/effector memory cells. CD69+ expression is a distinguishing early T cell activation marker, noted to initially increase shortly after TCR interaction, before slowly decreasing overextended culture, and is therefore indicative of T cell activation. PD-1 is a vital immune checkpoint receptor involved in immune-regulation. Activation of the PD-1 receptor by its corresponding ligand(PD-L1) promotes self-tolerance and immune regulation, suppressing T cell function. PD-1 is therefore not present on naive T cells but instead up regulation following expression and differentiation.

Thy1.1 and CD8 were primarily stained to ensure the analysed population was solely CL4 CD8+ T cells. Samples were stained for the 4 molecules, for 6 consecutive days following initial co-culture. Following daily staining the samples were fixed in 1% PFA, and all run on a Fortesa flow cytometer system. Results shown in figure 27.

Results show a sharp increase CD69 expression as soon as day 1, suggesting high levels of naive T cell activation and therefore successful priming. The level of CD69 is noted to peak on day 2, before beginning to descend until day 6 where it again increases, this could imply a re-stimulation

of a memory population within the culture. Furthermore, day 1 sees a large increase in PD-1 expression further supporting successful priming of naive T cells into CTLs. The level of PD-1 is sustained throughout the rest of the assay, demonstrating an active mature CTL population. A gradual increasing expression of CD44 over the first 2 days of culture is seen, depicting the activation of CTLs and loss of naive T cells. In contrast, CD62L is seen to sharply drop on day 1, further indicating the activation of naive T cells. However, CD62L expression is then seen to rise until Day 3 where it begins to again decrease. This may be advocating the differentiation of a central memory population of T cells on day 3. Central memory T cells are often identified as CD44+ CD62L+, in contrast to Effector/effector memory T cells which are commonly determined by their loss of CD62 expression. The molecular profile of the population on Day 3 suggests a high number of central memory T cells, primarily by the high level of both CD44 and CD62L. However, this population is seen to further differentiate from day 4 onwards where the gradual loss of CD62L suggests the formation of an increasingly effector/effector memory population.

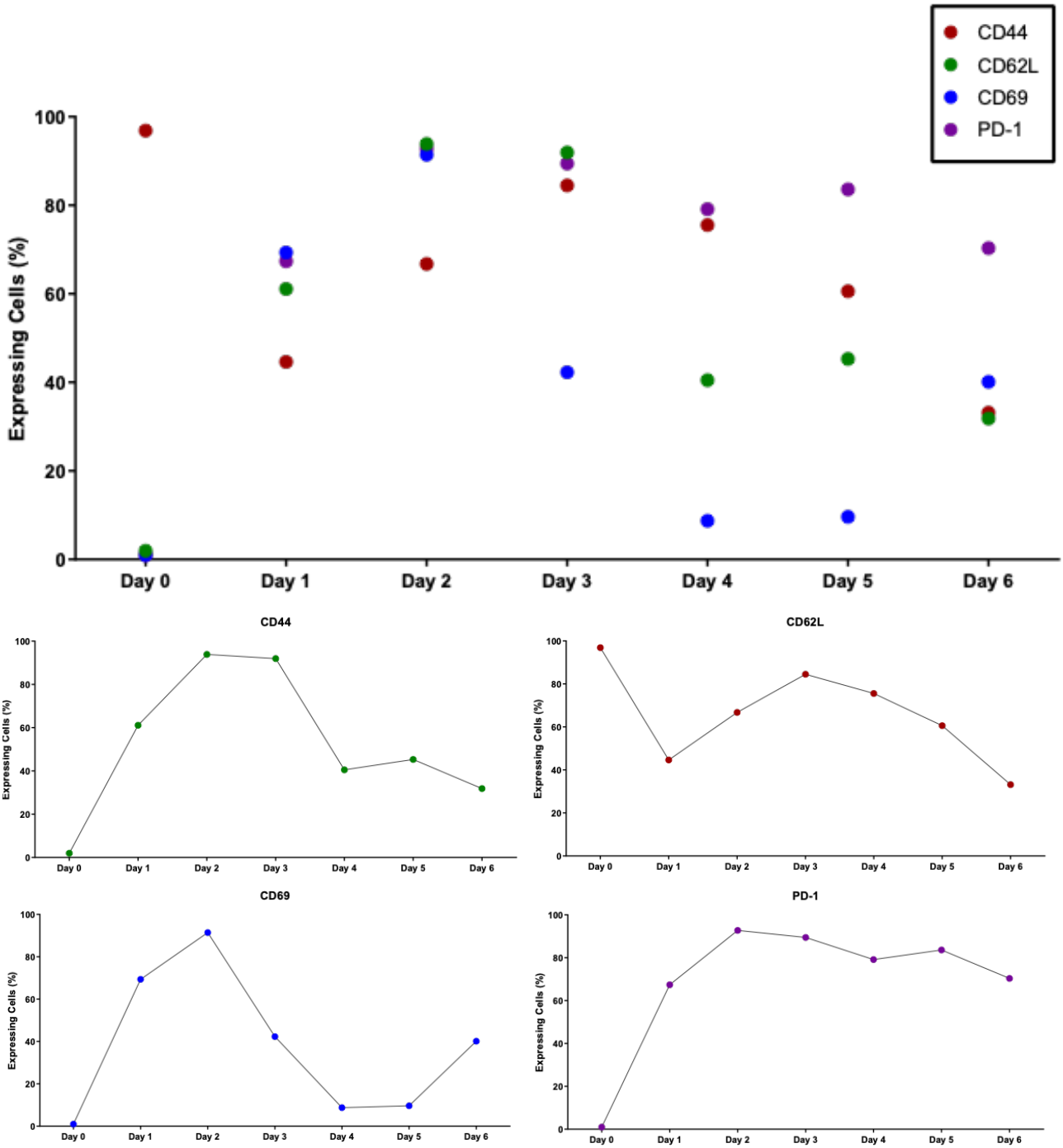


Figure 27: A time course of activation and differentiation markers

CL4-/- splenocytes were harvested and washed twice in RPMI. Cells were resuspended in 30mL T cell media and transferred into a 50mL Falcon tube. Cells were irradiated for 2500 seconds, 30cm away from the source, resulting in 3000 Rads. Once complete, splenocytes were washed twice and resuspended in 1mL T cell media and pulsed with 5uL K^dHA for 1hr. Naive CL4 splenocytes were harvested, counted and washed twice in RPMI. When complete, cells were resuspended at $5x10^5$ cells/mL in T cell media. CL4-/- splenocytes were washed 3 times in RPMI, counted and resuspended to $5x10^6$ cells/mL in T cell media. 1mL of both cell solutions were co-cultured together in wells of a 24 well plate and cultured for 144 hours. At time of priming and every 24 hours throughout the culture culture, 250uL cells was extracted from one well and transferred into FACs tubes. Samples were live/dead stained using Zmb NIR, using the previously described method. Once stained, cells were washed twice and extracellularly stained for CD44(PerCP-Cy5.5), CD62L(PE), CD69(BV605), and PD-1(BV786) using the protocol described in methods. Samples were fixed in 200ul 1% PFA and run within 1 week.

Results show on day 0 before priming, no naïve CTLs express CD44, CD69 or PD-1. In contrast, CD62L is expressed by almost 100% of naïve CTLs. Following priming on day one CD44, CD69 and PD-1 all increase dramatically to 61%, 70% and 69% respectively. Simultaneous, CD62L decreased to 43%. CD44 and CD69 both peaked population expression at 97% and 98% respecifully, on Day 2, followed by a continuous steep decrease over the remaining 4 days. PD-1 also peaked on day two at 98%, where levels maintained a shallow decrease for the remaining 4 days. CD62L began to rise following day 1 until day 3 where it peaked at 84%. From day 3 CD62L decreased over the remaining assay.(n=2)

3.3.5 Are CTLs able to effectively eliminate cancer cells when cultured in the absence of IL-2?

Cytotoxic T Cells play a key role in preventing cancer development via immunosurveillance. Supplementary exogenous IL-2 added to culture media increases the survival and cytotoxic capabilities of CTLs making them extremely effective killers. However, the high concentrations of supplementary IL-2 proves un-physiological and therefore not representative of in vivo studies. Previous studies have suggested the role of IL-2 in the prevention of suppression following treatment via PGE2. To overcome the limitation of in vitro IL-2 supplemented CTL studies the newly developed CTL culture method utilises no additional IL-2, instead uses solely antigen presenting cells and CL4 splenocytes, deducing a more physiological method of priming.

In order to determine whether the CTLs are able to eliminate cancer cells when cultured in the absence of IL-2, imaging-based kill assays were completed. CTLs were cultured for 96 hours using the novel culture method previously described and therefore requiring no exogenous IL-2 other than a result of autocrine/paracrine production. Renca cells, previously transfected with the mCherry plasmid(Renca mCherry), emit a red cytoplasmic fluorescence allowing their detection by the IncuCyte incubated microscope. MCherry Renca cells were plated into a glass bottomed 384 well plate and incubated until adherence to the plate occurred. Once the mCherry cells had adhered the new cultured CTLs were harvested and sorted for live cells using fluorescence-activated cell sorting(FACs). Sorting ensured that equal numbers of live CTLs would be co-cultured. The CTLs were able to be sorted solely on live cells as over 85% of the remaining alive cells following 96

hours of culture were CL4 CD8+ cells, demonstrated previously. Once sorted, equal numbers of CTLs were added onto of the wells containing adhered mCherry Renca cells at a 1:1 ratio. The plate was then incubated for 18 hours in an IncuCyte live cell analysis system(Essenbioscience). Upon completion of the assay, total surface area of mCherry cells(uM2/well) were calculated and averaged across 4 repeats. Resulting data was normalised and converted into rolling averages to enhance the overall trend. Decreases in the surface area of mCherry cells was due to cell death and was indicative of cytolysis via CTLs. Un-pulsed Renca cells were used as a control to ensure the cytolysis seen was specific towards cells presenting the HA antigen and therefore completed by CL4 CTLs. In addition, wells of solely Renca cells were run in order to consider the natural increase in surface area over 17 hours due to Renca cell proliferation.

Results, seen in figure 28, show a significant decrease in the level of cytolysis exerted by CL4 CTLs primed in the absence of IL-2. Significant cytolysis is seen by CL4 CTL cells cultured in the absence of IL-2. Yet, the steep decreasing gradient depicting IL-2 CL4 CTL cytolysis, demonstrates a dramatically enhanced cytotoxic ability. Further gradient comparisons, shown in figure 29, clearly show the decreased effector function of CTLs primed in the absence of IL-2. However, the increased ability of IL-2 CL4 CTLs is not necessarily physiological, and may been unrealistically effective. Further investigations must be completed to determine the correct physiological rate of CTL cytolysis. Overall effective cytolysis was witnessed in the newly cultured CTLs, therefore future experiments into the suppressive effects of PGE2 on CTL effector function were able to utilise the new culture method circumventing the necessity for increased IL-2 supplementation.

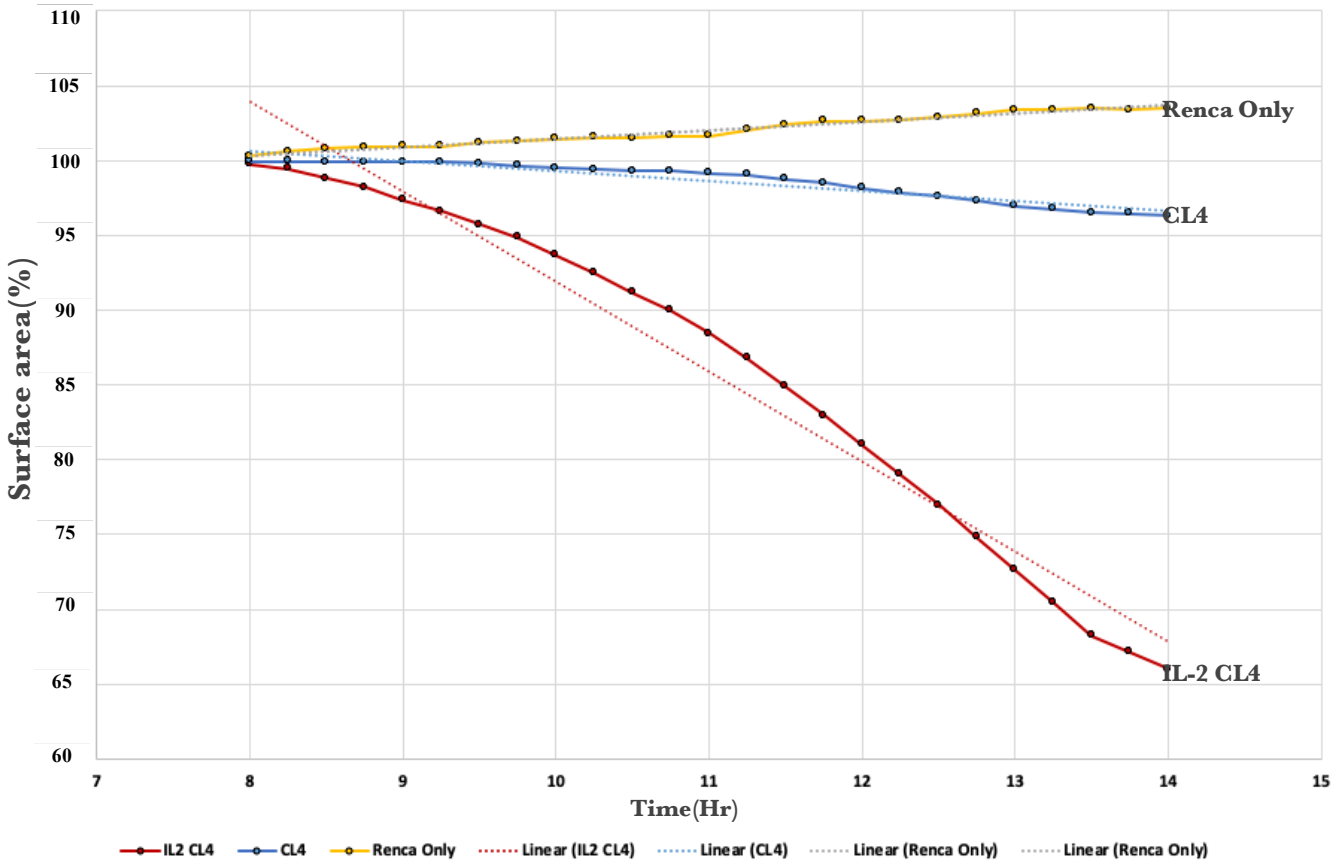


Figure 28 : Imaging based kill assay to compare the cytolytic effect of CTLs when culture +/- supplementary IL-2

CL4$^{-/-}$ splenocytes were harvested and washed twice in RPMI. Cells were resuspended in 30mL T cell media and transferred into a 50mL Falcon tube. Cells were irradiated for 2500 seconds, 30cm away from the source, resulting in 3000 Rads. Once complete, splenocytes were washed twice and resuspended in 1mL T cell media and pulsed with 5uL K^{d}HA for 1hr. Naive CL4$^{+/-}$splenocytes were harvested, counted and washed 3 times in RPMI. 4x10^{6} CL4$^{+/-}$ splenocytes were extracted from the total resuspended at 5x10^{5} cells/mL in T cell media. CL4$^{-/-}$ splenocytes were washed 3 times in RPMI, counted and resuspended to 5x10^{6} cells/mL in T cell media. 1mL of both CL4$^{-/-}$ and CL4 $^{+/-}$ solutions were co-cultured in 8 individual centre wells of a 24 well plate, and left to culture for 96 hours. The remaining naive CL4$^{+/-}$ splenocytes were primed overnight using a mixed lymphocyte reaction with K^{d}HA, as described in methods. 5x10^{6} primed splenocytes per condition were harvested, washed and resuspended in 2ml IL-2 media with their corresponding conditions. The cellular suspensions were plated into separate wells of a 24 well plate and cultured for an additional 72 hours. 60-80% confluent Renca mCherry cells were detached from a T25 flask using 500uL Trypsin-EDTA. Detached cells were quenched in 4mL complete media, collected into a 15mL falcon tube and centrifuged. Renca mCherry cells were resuspended in 1mL complete media and counted. Additional complete media was added until 1x10^{6} cells/mL was achieved. 2uL K^{d}HA was added to the suspension, and incubated for 60 minutes, at 37^{o}C. Cells were centrifuged, supernatant discarded and washed twice using 10mL RPMI. Once washed, Renca mCherry cells were re-counted, and resuspended to 3x10^{5} cells/mL in Fluorobrite media. 50uL Renca mCherry cells were plated in individual wells of a glass bottomed 384 well plate, and incubated until cellular adherence, at 37^{o}C. 4 repeats per condition were plated. All cultured CTLs were harvested into separate 15mL falcon tubes corresponding to their culture conditions. All CTLs were washed in PBS, and resuspended in 1mL conditioned imaging buffer. 1.5mL eppendorf were prepared containing 200uL imaging buffer, for each experimental condition.

CTLs were sorted using FACs for 60,000 alive cells per condition into their corresponding eppendorf, taking into account an un-pulsed control group. Sorted CTLs were centrifuged, 4000xg 4minutes, using a microfuge, and supernatant carefully extracted ensuring no disturbance to the cellular pellet. All CTLs were resuspended to $3x10^5$ cells/mL in Fluorobrite media. 50uL CTLs were added to corresponding target wells containing adhered Renca mCherry, immediately before the plate was placed in the IncuCyte imaging system for 18 hours. Total target cell surface area (um^2/well) was measuring using red fluorescence masking, over the duration of the assay. The mean of 4 repeats was calculated per timepoint for each condition. Results were normalised, and rolling averages produce. A row of Renca only controls were used to demonstrate cell growth over the duration of the assay. The decrease in target cell surface area was indicative of target cell death via CTL cytolysis. The results show a significant increase in the rate of cytolysis induce by IL-2 supplemented CL4 (red) compared to the CL4 cultured using the newly developed culture method . (n=1)

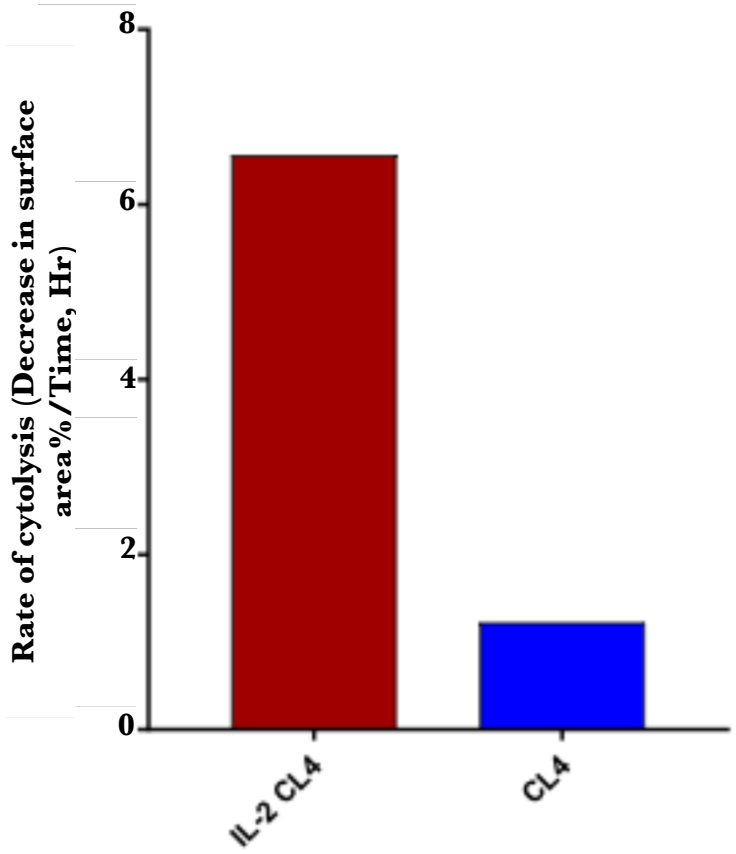


Figure 29: Comparative rates of CTL induced target cytolysis.

The gradient of each kill assay time curve was calculated by division of the change in surface area by time. The gradient of the increase in surface area demonstrated by the Renca only control, was detracted from the resulting cytolytic gradients to account for the full change in surface are and therefore cytolysis over the time course. The results gradients were plotted on a bar chart to represent the rate of cytolysis. A T test confirmed a significant difference in the rate of cytolysis between CL4 cultured using the newly developed culture method and IL-2 supplemented culture.

3.3.6 Does PGE2 suppress CTL proliferation when cultured in the absence of IL-2?

The previous study demonstrated that following 72 hour culture in the absence of Il-2, 1uM PGE2 significantly suppressed the level of proliferation seen, indicating the role of PGE2 in tumour mediated immune suppression. The previously used culture method the population of CD8+ T cells within the control group, in the absence of supplementary IL-2, was not able to be efficiently utilised for further investigations into the suppressive effects of PGE2, due to both timing and limited resources. However, the newly developed method of priming enables high levels of surviving CL4 CD8+ T cells following 96 hours in culture. In order to confirm that the previous 1uM PGE2 induced suppression witnessed, was not solely relevant when used in combination with the previous culture methods, additional proliferation assays were complete to determine the suppressive effect of 1uM PGE2.

CTV stained CL4 splenocytes were primed and cultured for 96 hours in the presence of 1uM PGE2. Once complete the cells were Live/Dead stained, and stained for Thy1.1+ and CD8+ surface molecules to enable identification of CL4 CD8+ T cells. The level of proliferation was determined by examining the percentage of cells that reached the final two division through the culture.

Results, seen in figure 30, show a significant reduction in the proliferation of CL4 CD8+ T cells following the addition of 1uM PGE2. 48% of PGE2 treated CTLs were seen to reach the final two proliferative events, compared to 78% of the untreated control. Parametric T tests confirmed the suppressive effect seen to be significant. The suppressive effect of 1uM pGE2 in the absence of additional IL-2 is therefore not restricted to the type of T cell priming. The combined investigations thereby support currently literature demonstrating a suppression of proliferation, in CTLs priming using irradiated Renca cells in the presence of 1uM PGE2.

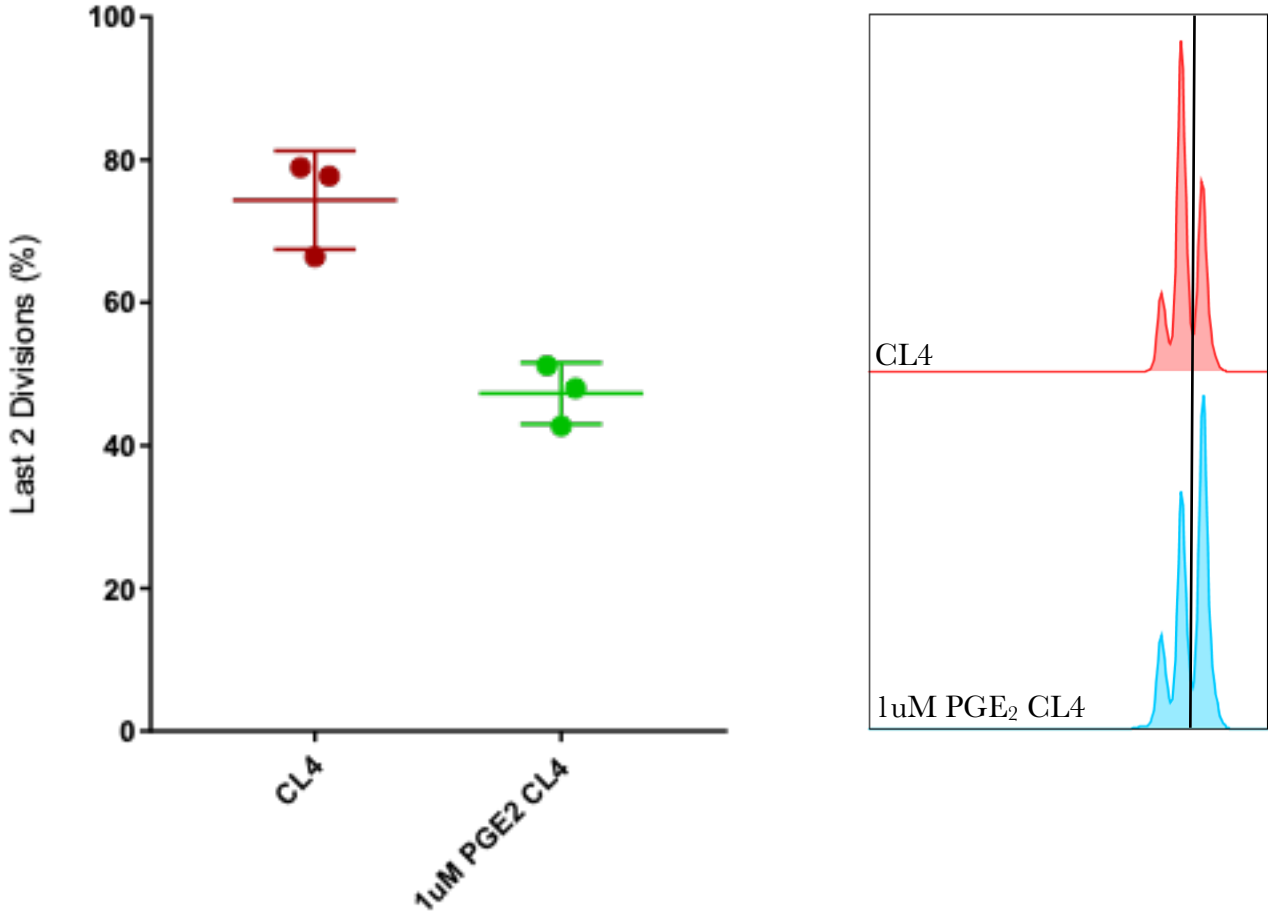


Figure 30: Effect of PGE2 on CL4 CTL proliferation

CL4-/- splenocytes were harvested and washed twice in RPMI. Cells were resuspended in 30mL T cell media and transferred into a 50mL Falcon tube. Cells were irradiated for 2500 seconds, 30cm away from the source, resulting in 3000 Rads. Once complete, splenocytes were washed twice and resuspended in 1mL T cell media and pulsed with 5uL K^dHA for 1hr. Naive CL4 splenocytes were harvested, counted and washed twice in PBS. CTV stain was diluted 1uL in 1000uL PBS. 1uL CTV was used per $4x10^6$ cells. Splenocytes were centrifuged, supernatant discarded and resuspended in diluted CTV stain. CTV Staining was completed at 37^{oC} for 15 minutes, followed by a 5x quench in 0.5% protein rich media and an additional 15 minute, dark incubation at room temperature. When complete, cells were washed 3 times in RPMI, re-counted and resuspended at $5x10^5$ cells/mL in T cell media. CL4-/- splenocytes were washed 3 times in RPMI, counted and resuspended to $5x10^6$ cells/mL in T cell media. 1mL of both cell solutions were co-cultured together in wells of a 24 well plate. Wells were either treated with 1uM PGE_2 or a vehicle control and cultured for 96 hours. Following culture, 250uL cells was extracted from each well and transferred into individual FACs tubes. Samples were live/dead stained using Zmb NIR, using the previously described method. Once stained, cells were washed twice and extracellularly stained for both CD8(APC) and Thy1.1(FITC), using the protocol described in methods. Samples were run on a Novocyte flow cytometry system, gated strategy in figure 18. CTV fluorescence was used to determine the number of cellular division and the percentages of cells reaching the last two divisions were calculated. The results show a significant reduction($n=1$, $p<0.0045$) in the percentage of CL4 CTLs reaching the last two divisions when treated with 1uM PGE_2.

3.3.7 Does PGE2 suppress CTL cytolytic function when cultured in the absence of IL-2?

PGE2 has been demonstrated to exert an immunosuppressive effect on the proliferation of CTLs when incorporated into culture. The suppression seen has been demonstrated to be independent on the method of priming, both direct priming via tumour cells and cross presentation via APCs are seen to be suppressed upon the addition of 1uM PGE2. However, the suppression of proliferation at 1uM PGE2 is only witnessed when no supplementary IL-2 is added to the culture. The addition of IL-2 in culture media has been seen to inhibit suppression and increase the concentration of PGE2 required to exhibit any suppressive effects.

When CTLs are cultured in high levels of IL-2, previously described, the addition of 1uM PGE2 is not seen to significantly inhibit CTL cytolysis. To determine whether the lack of effect of 1uM pGE2 previously seen were true of due to high levels of IL-2 in the culture media, further kill assays were completed using both: CTLs primed using the previous method of culturing without supplementation of IL-2, in addition to CTLs primed using the new culture method(+/- 1uM PGE2).

In order to confirm the effects of PGE2 on CTL cytolysis in the absence of additional IL-2, the well-established and controlled incucyte imaging-based killed assay was completed. Using an imaging-based kill assay allowed for the identification of any confounding factors that may alter the conclusion of results, ensuring the affects occurring were correct. IncuCyte kill assays were set up, as previously described, using Renca mCherry cells as targets. CTLs were primed using either the Wuelfing lab culture method without the protocoled addition of IL-2, or using the newly developed culture method. Either 1uM PGE2 or a vehicle control was added at the time of priming. Results were acquired in the form of rate of change in surface area. The total surface area of cytoplasmic red florescence of mCherry cells per well was calculated using IncuCyte Zoom analysis. Once recorded the resulting data was normalised and rolling means calculated to represent the overall

directory, shown in figure 31. Gradients of each condition were calculated over representative time points and compared to demonstrate the level of cytolytic function, as seen in figure 32.

The results show a significant reduction in the rate of cytolysis exhibited by CTLs when cultured in 1uM PGE2, in the absence of additional IL-2. 1uM PGE2 is seen to completely inhibit the ability of CTLs to eliminate mCherry renca cells. No decrease in surface area is witnessed when 1uM PGE2 treated CTLs are co-cultured with HA presenting mCherry cells, clearly demonstrating significant suppression of cytolytic function. This is confirmed through analysis of the rate of cytolysis as depicted by gradient of the slope. A negative gradient is noted in the results, presented as 0, deeming complete inhibition of cytolysis.

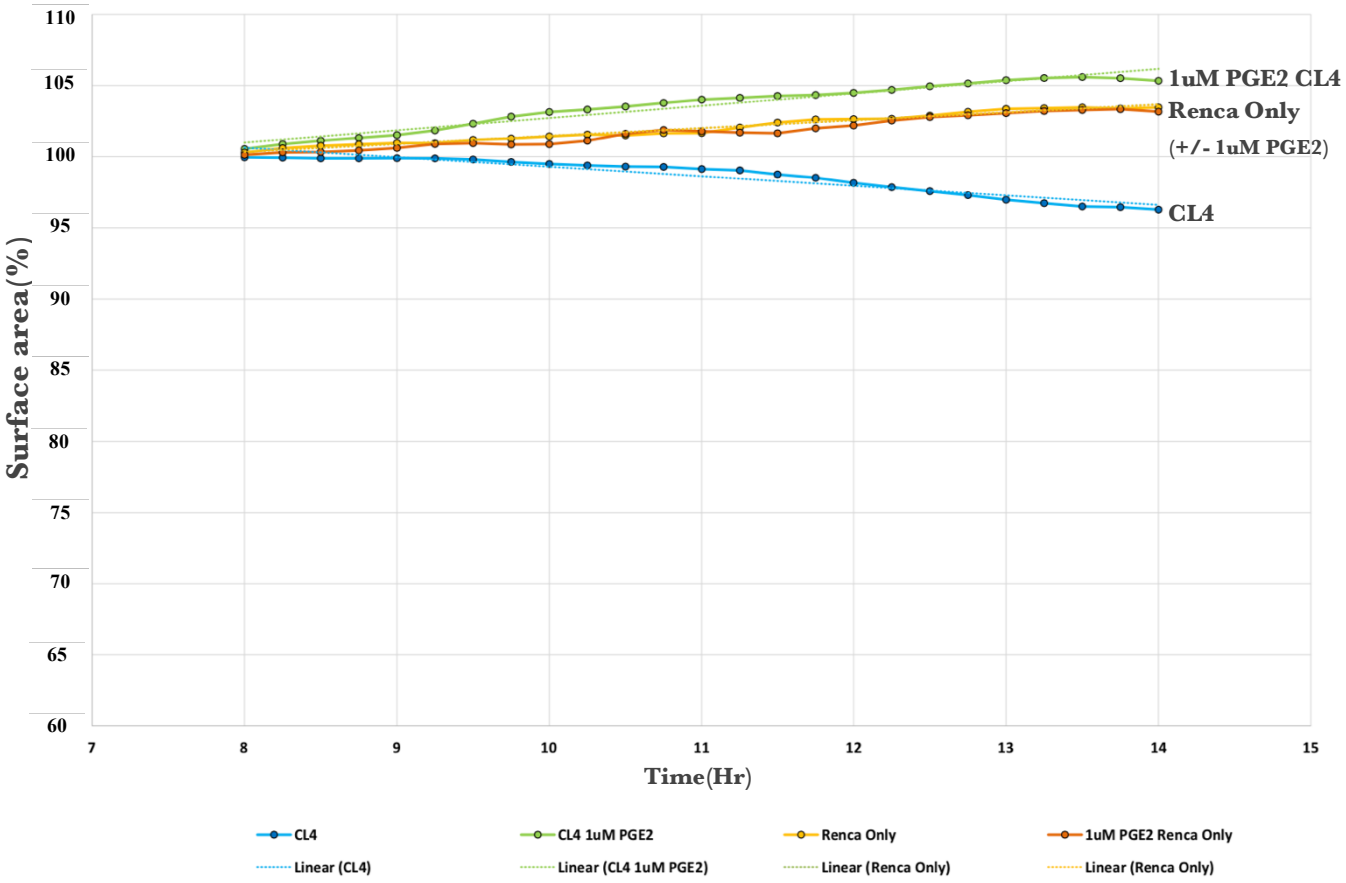


Figure 31: Imaging based kill assay to compare the suppressive effect of 1uM PGE2 on CTLs cytotoxic function.

CL4$^{-/-}$ splenocytes were harvested and washed twice in RPMI. Cells were resuspended in 30mL T cell media and transferred into a 50mL Falcon tube. Cells were irradiated for 2500 seconds, 30cm away from the source, resulting in 3000 Rads. Once complete, splenocytes were washed twice and resuspended in 1mL T cell media and pulsed with 5uL K^{d}HA for 1hr. Naive CL4$^{+/-}$splenocytes were harvested, counted and washed 3 times in RPMI. Cells were resuspended at 5x10^{5} cells/mL in T cell media. CL4$^{-/-}$ splenocytes were washed 3 times in RPMI, counted and resuspended to 5x10^{6} cells/mL in T cell media. 1mL of both CL4$^{-/-}$ and CL4 $^{+/-}$ solutions were co-cultured in 8 individual centre wells of a 24 well plate. Select wells were treated with 1uM PGE_2 or a vehicle control and cultured for 96 hours. 60-80% confluent Renca mCherry cells were detached from a T25 flask using 1mL Trypsin-EDTA. Detached cells were quenched in 4mL complete media, collected into a 15mL falcon tube and centrifuged. Renca mCherry cells were resuspended in 1mL complete media and counted. Additional complete media was added until 1x10^{6} cells/mL was achieved. 2uL K^{d}HA was added to the suspension, and incubated for 60 minutes, at 37^{o}C. Cells were centrifuged, supernatant discarded and washed twice using 10mL RPMI. Once washed, Renca mCherry cells were re-counted, and resuspended to 3x10^{5} cells/mL in Fluorobrite media. 50uL Renca mCherry cells were plated in individual wells of a glass bottomed 384 well plate, and incubated until cellular adherence, at 37^{o}C. 4 repeats per condition were plated. All cultured CTLs were harvested into separate 15mL falcon tubes corresponding to their culture conditions. All CTLs were washed in PBS, and resuspended in 1mL conditioned imaging buffer. 1.5mL eppendorf were prepared containing 200uL 2X conditioned imaging buffer, for each experimental condition. CTLs were sorted using FACs for 60,000 alive cells per condition into their corresponding eppendorf, taking into account an un-pulsed control group. Sorted CTLs were centrifuged, 4000xg 4minutes, using a microfuge, and supernatant carefully extracted ensuring no disturbance to the cellular pellet. All CTLs were resuspended to 3x10^{5} cells/mL in 2X conditioned Fluorobrite media.

50uL CTLs were added to corresponding target wells containing adhered Renca mCherry, immediately before the plate was placed in the IncuCyte imaging system for 18 hours. Total target cell surface area(um²/well) was measuring using red fluorescence masking, over the duration of the assay. The mean of 4 repeats was calculated per timepoint for each condition. Results were normalised, and rolling averages produce. A row of Renca only controls were used to demonstrate cell growth over the duration of the assay. The decrease in target cell surface area was indicative of target cell death via CTL cytolysis. The results show a significant increase in the rate of cytolysis induce by IL-2 supplemented CL4(red) compared to the CL4 cultured using the newly developed culture method.(n=1)

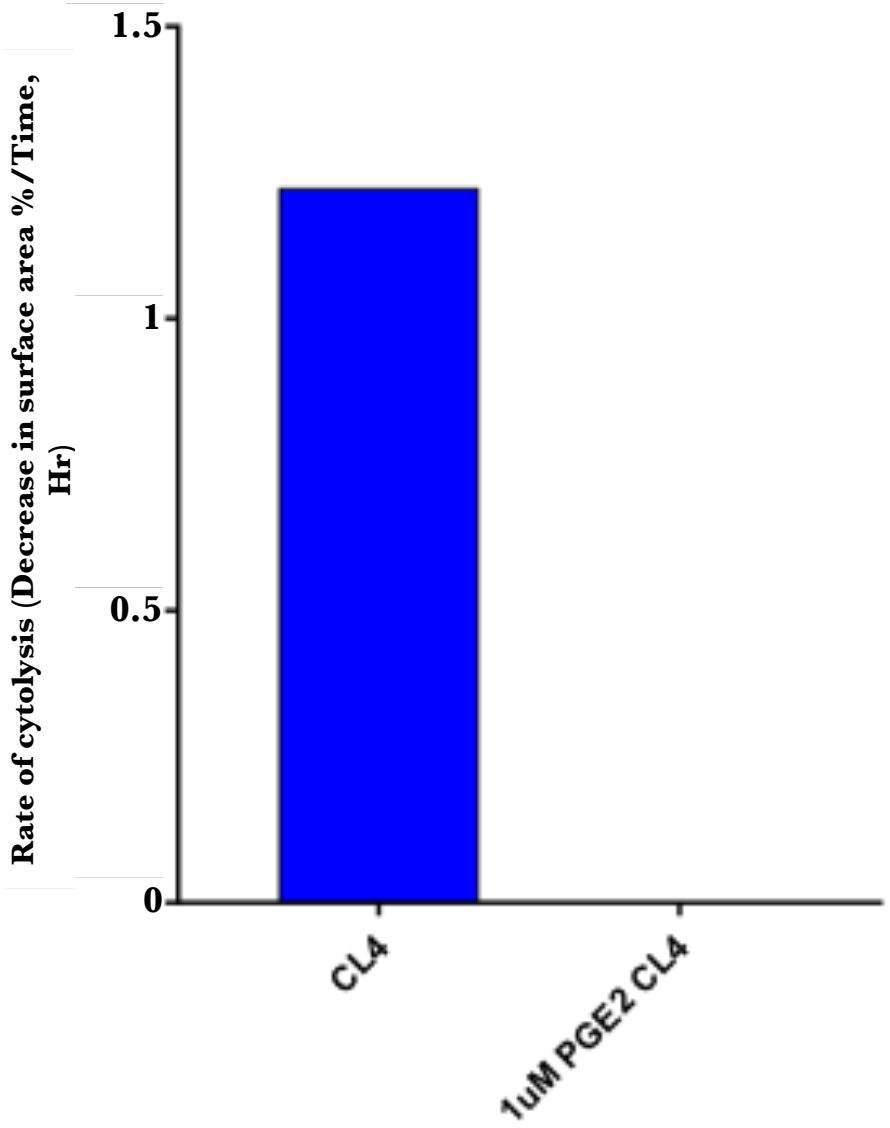


Figure 32: Effect of PGE_2 on CTL induced target cytolysis.
The gradient of each kill assay time curve was calculated by division of the change in surface area by time. The gradient of the increase in surface area demonstrated by the renca only control, was detracted from the resulting cytolytic gradients to account for the full change in surface are and therefore cytolysis over the time course. The results gradients were plotted on a bar chart to represent the rate of cytolysis. A T test confirmed a significant reduction in the rate of cytolysis following culture in the presence of 1uM PGE_2.

3.3.8 Discussion

The newly developed method for priming and culturing T cells abolishes the need for supplementary IL-2. The addition of high levels of IL-2 in the previous culture method is unphysiological and has been proven to largely inhibit the level of suppression seen in CTLs when exposed to PGE2. Previous studies using the culture method for adoptive transfer models in both mice and tumour spheroids, have witnessed extreme levels of suppression in both synapse maintenance and cytolysis(135). However, the CL4 CTL controls have been consistently cultured in a replenished media containing high levels of IL-2 compared to the adoptively transfer samples that have not only entered a solution with much lower levels of IL-2 but also the additional investigated suppressive mediators within the tumour microenvironment. In contrast, the in vitro studies reported in the previous chapter, unexpectedly saw little/no suppression when cultured with varied levels of PGE2 contrasting previous studies which have demonstrated 1uM PGE2 to be sufficient in order to suppress both the proliferation and IFN-y production when priming naive T cells(91). This effect was not originally witnessed using the previous culture method, only showing significant suppression when a 10-fold higher concentration of PGE2(10uM) was used, in addition to no significant suppression of cytolysis being witnessed. The lack of suppression was believed to primarily been due to the high level of IL-2 in the culture media. The newly developed method for priming and culturing T cells abolishes the need for supplementary IL-2, making a much more physiologically relevant way of priming and culturing CTLs.

The new culture method has shown to be extremely simplistic and manageable, preventing the need for bi-daily media changes over the weekend. In addition, the high percentage of CL4 CD8+ T cells by 96 hours, allows for efficient fluorescence-activated cell sorting of CL4 CTLs merely on gating for cells expressing Thy1.1+ or stringent gating on live cells. Being able to isolate a pure CL4 CD8+ population by gating solely on live cells or even Thy1.1+ is essential for the efficient use of CL4 CTLs in kill assays, as staining for molecules such as CD8 or TCR has the

potential to block or stimulate the molecules potentially inducing aberrant effects, therefore affecting the accuracy of the results.

Further characterisation of the cell surface molecules: CD44, CD62L, CD69 and PD-1, clearly demonstrated successful activation of naive T cells throughout the cell culture. Population molecular profiles showed successful, physiologically relevant activation and differentiation of T cells following the new IL-2 abrogated culture method. By day 3 it was noted that a population of central memory T cells had developed, only to further differentiate into effector and effector memory T cells over the following consecutive days. The formation of an effector/effector memory population seen on day 4 indicates it to be the preferred earliest time for CTL use in kill assays, as it is effector/effector memory cells that shall best induce a cytolytic effect. Day 6 would be an ideal time to use CTLs in a kill assay because of the highest percentage of effector cells, however by day 6 lower levels of surviving cells was noticed, therefore further optimisation should be completed in order to fully maximise the potential of the new culture method.

IL-2 heavily induces T cell differentiation and proliferation, hence its use in both cytokine therapy and expanding T Cell populations for adoptive T cell transfer(197). The previous culture method demonstrated the use of IL-2, with much higher levels of proliferation being seen in CD8+ T cells primed using a single spleen mixed lymphocyte reaction upon the addition IL-2. However, the new culture method was seen to be capable of inducing equally comparable levels of proliferation over 96 hours without the need for supplementary IL-2, further demonstrating a more physiological and effective method of priming naive T cells. In addition, when testing the effect of 1uM PGE2 of the priming of CTLs using the new culture method, suppression was seen.

An additional function of IL-2 is to encourage the production of both IFN-y and the key molecules used in CTL effector function, perforin and granzymes(195). IL-2 signalling induces multiple intracellular signalling pathways including the activation of mTORC1 signalling, in turn encouraging the production of pro inflammatory molecules. The production of both IFN-y and the key molecules used in CTL effector function, perforin and granzymes has been noticeably increased

following IL-2 culture(195). Increased IL-2 is also heavily associated with differentiation of effector CTLs abolishing the population of memory CTLs. The increase in pro-inflammatory mediator production following IL-2 exposure is noted when comparing the cytolytic ability of CTLs cultured using the previous method of culture to that of the new. CTLs cultured in absence of IL-2 demonstrated a strong cytolytic ability against tumour cells, however CTLs cultured in IL-2 showed dramatically enhanced killing ability, eliminating tumour cells at a much higher rate. The increased rate of killing could also be due to an increased population of memory T cells in the CTL population of cells in absence of IL-2.

However, this is merely speculation as CTLs cultured using the old, IL-2 supplemented, culture method have not yet been characterised. Therefore, for further investigation into to differing cytotoxic abilities of the CTLs resulting from differing culture methods(+/- IL-2) further in depth comparable characterisation should be completed.

Investigations into the effect of PGE2 on CTLs were completed using the newly developed CTL culture to acquire more physiological and representative results of potentially suppressive mechanisms within the tumour microenvironment. Previous investigations showed that 1uM PGE2 was sufficient to suppress the proliferation of CTLs, however that the effect was only noted in the absence of supplementary IL-2. Repeated proliferation assays using the new culture method further confirmed this finding, showing a reduced capacity of proliferation in CTLs culture with 1um PGE2. The results further supported the study and previous findings, suggesting that PGE2 could have a role in the suppression of CTL proliferation following metastasis of tumour cells into tumour draining lymph nodes. Following metastasis of tumour cells into the lymph nodes, directly activated tumour specific CTLs would then home to the tumour in order to eliminate cancer cells. Previous studies into the effector function of CTLs primed in the presence of PGE2, showed no significant findings however this was believed to be due to the high levels of additional IL-2. The new culture method enabled investigation into the effect of PGE2 in the absence of supplemented IL-2. Results showed a complete inhibition in cytolysis of tumour cells by CTLs primed and cultured in 1uM

PGE2. This clearly demonstrates not only, that IL-2 has the ability to circumvent suppression seen by PGE2, but that 1uM PGE2 may be an important mechanism utilised and unregulated by cancer cells to suppress the cytotoxic function of CTLs and therefore contribute to tumour development.

3.4 Characterisation of new cancer cell lines

3.4.1 Introduction

In vitro cancer studies provide a useful tool for investigations into understanding the intricate interactions between cancer development and progression and the immune system. Multiple well-established cancer cell lines have been developed and optimised for use in studies, one prime example being the murine renal carcinoma model, Renca. Replicative immortality, coupled with advanced long-term liquid nitrogen storage, enables the extended use of many cancer cells lines for many years, enabling a multitude of experiments and research to be undertaken. However, cancer is recognised as not a single but instead a group of over 200 different diseases due to the multifaceted phenotypic and genetic alterations seen in varying cancer types. The vast variation in cancer types is one of the reasons developments in treatments and prognoses proves so difficult. For translational research to occur, studies must investigate multiple cancer types to determine whether effects witnessed are phenotypic of a broad class of cancers or merely the single cell line being investigated. Therefore, the development of two new murine cancer cell line models was initiated.

4T1 and CT26 are two new cancer cell lines in the Morgan lab. 4T1 is a cancer cell line originating from the mammary gland tissue of a mouse(181). Used primarily as model for breast cancer, the 4T1 cell line is epithelial and has been reported to metastasis within murine models(181). 4T1 cells are extremely proliferative, dividing at very high rates, and results in cellular extrusion and apoptosis following 100% confluence in culture. In vivo tumours are made up of a mixture of multiple cell lines at different ratios depending on the type and origin. Therefore, in addition to the new epithelial 4T1 cell line, a fibroblastic cell line derived from a murine model colorectal carcinoma was developed, named CT26. Enabling the completion of future studies on a variety of cancer cell types and origins will allow for the identification of additional cancer-induced immune effects, whilst determining whether they are cancer cell specific or translational(180).

3.4.2 Aims

I. To characterise the cell surface molecular patterning

II. To determine whether 4T1 and CT26 cells are capable of priming naïve CL4 T cells.

III. To determine whether CL4 CTLs are able to eliminate 4T1 and CT26

IV. To transfect the 4T1 and CT26 cell lines to express the HA peptide.

3.4.3 Characterisation of cell lines

In order to utilise new cancer cell lines in research it must be determined which key cell surface molecules they express, particularly for immunological studies. For cancer cells to interact with cytotoxic T cells they must be capable of both being recognised and providing secondary co-stimulation(87). The interactions between CTLs and cancer cells is increasingly complex. CD8+ T cells are primed and activated through two main signals. The first is recognition of a specific antigen bound to class 1 MHC complex. However ultimately the cancer cell co-signalling receptor expression determines the fate of the CD8+ T cell. A wide variety of co-signalling receptors, both stimulatory and inhibitory, have been identified to affect CTL function and activation. In order to effectively use the new 4T1 and CT26 cancer cell lines, their cell surface molecular expression patterning was first characterised, in attempt to identify key classical immunological receptors.

To characterise the cell surface molecular expression of both the 4T1 and CT26 cell lines, cells were stained using fluorochrome conjugated antibodies, each specific for a cell surface receptor. The chosen cell surface molecules stained for were: MHC1, MHC2, CD80, CD86, CD40, ICAM-1, PD-1, and Galectin-9. Samples of each cell line were stained with single stains enabling the detection of the population expression of individual specific cell surface molecules, depicted by a positive fluorescence population shift. Comparisons between unstained cells and sings stained samples allowed for detection of individual molecule expression on the cell surface. All

antibodies were titrated, for optimal use and ensuring accurate results. Both accutase and Trypsin were tested and compared when detaching the cells from culture preceding staining, to ensure no molecules were undetected due to cleavage by trypsin.

MHC1 is expressed on almost all nucleated cells of the body. It is used to present fragments of degraded intracellular peptides, enabling detection of viral infection or transformation by the immune system. MHC1 is the main signaling complex in CTL interaction. The specific TCR binds to MHC1 complexes to both stimulate priming and proliferation, as well as emitting their cytotoxic effector function. In order to utilise a tumour cell line for immunological studies, it is therefore essential that they express MHC1 to allow interactions with CTLs. However, cancer cells have been reported to down regulate MHC1 complex in attempt to evade detection by the immune system(198).

MHC2 is primarily expressed on professional antigen presenting cells of the immune system. Phagocytotic cells such as Dendritic cells are most commonly associated with MHC2 as they phagocytose antigenic peptides in order to present them, via MHC2, to additional immune cells within the lymphatic system. Therefore, MHC2 is not expected to be expressed on the cell surface of epithelial or fibroblast cancer cells.

For successful T cell stimulation, CTLs must receive two independent signals. First, the TCR must recognise and interact with an MHC1 bound antigen. The second signal must come from a co-stimulatory molecule. Traditionally CD80 and CD86 were recognised and the two primary CTL co-signaling molecules(87). Binding to CD28, the presence of one or both molecules enables sufficient T cell signaling inducing proliferation and effector function. More recently the role of ICAM-1 has also been highlighted. Typically regarded for its role in leukocyte extravasion, ICAM-1 is also capable of inducing secondary stimulatory signals to CTLs(92). Furthermore, CD40 is a co-stimulatory molecule, however it is typically only present on professional antigen presenting cells. For a successful immunogenic tumour model at least one co-stimulatory molecule must be expressed on the cell surface to allow for effective CTL interactions.

Upon activation CTLs have been demonstrated to up regulate the Programmed death receptor-1(PD-1) expression on their cell surface(199). PD-1 is a regulatory protein, involved in eliminating exhausted CTLs in addition to preventing autoimmune disease by inducing self-tolerance through the inhibition and apoptosis of CTLs. PD-1 is activated through interaction with its ligand(PD-L1). Studies have reported cancer cells to utilise this immune-regulatory process in attempt to evade elimination by the immune system(199). Lymphocytes have been noted to have an increased expression of the PD-1 receptor following tumour infiltration, increasing their potential interaction and therefore suppression and cell death(200). Further studies have seen tumour cells over express PD-L1, further driving the potential of PD-1 dependent apoptosis.

Recent studies have noted cancer cells to up regulate an additional molecule capable of modulating T cell responses, named Galectin-9. Galectin-9 is a carbohydrate binding protein, expressed on both epithelial and endothelial cells in addition to a variety of immune cells, including B and T cells. Immune modulation via, galectin-9, is due to its ability to act as a stimulatory ligand for the Tim-3 receptor expressed by T cells. Tim-3 is an immune checkpoint and therefore co-inhibitory receptor of T cell function. Primarily involved in T cell homeostasis, and prevention of autoimmune disease, Tim-3 expression is noted in exhausted CTLs following prolonged antigen exposure. However increased activation of Tim-3, through the up regulation of Galectin-9 has been reported to be utilised by cancer cells as an evasive mechanism(201). Therefore, it is important to determine whether expression of both PD-L1 and Gal-9 is apparent on both new cell lines before immunological studies are completed.

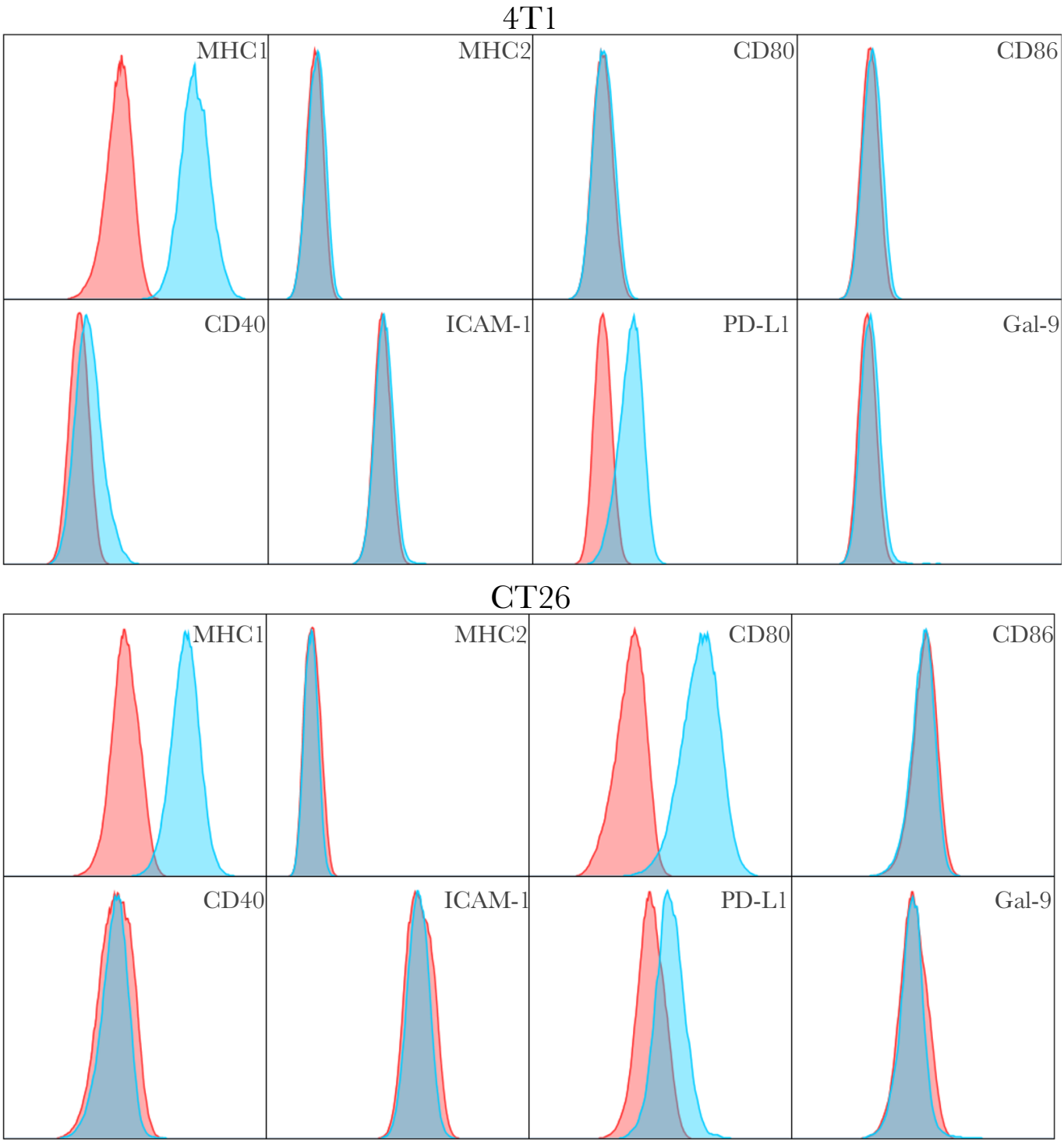


Figure 33: Cell line expression of key co-signalling molecules.
60-80% confluent Renca mCherry cells were detached from a T75 flask using 1mL Trypsin-EDTA. Detached cells were quenched in 4mL complete media, collected into a 15mL falcon tube and centrifuged. Renca mCherry cells were resuspended in 1mL complete media and counted. Additional complete media was added until $5x10^5$ cells/mL was achieved. 9 x 1mL samples were extracted into individual FACs tubes and washed twice in PBS. Samples were live/dead stained using Zmb NIR, using the previously described method. Once stained, one FACs tube was fixed to be used as a Zmb only control. Remaining samples were washed twice and each extracellularly stained, using the protocol described in methods, for a single molecule: MHC1(Pacific blue), MHC2(APC), CD80(BV650), CD86(BV605), CD40(PE), ICAM-1(FITC), PD-L1(BV786) and Galectin-9(BV421).

Samples were fixed in 200ul 1% PFA and run within 1 week. Both CT26 and 4T1 cells were shown to express Class 1 MHC complexes, shown by a right-ward population shifts in fluorescence emission. 4T1 cells were shown to not express any of the targeted co-stimulatory molecules(CD80, CD86, ICAM-1) however does express PD-L1. CT26 cells were seen to express CD80, in addition to PD-L1. Neither cell lines expressed MHC2, CD40, CD86, ICAM-1 or Galectin-9.(n=6)

Results shown in figure 30 show a clear population shift increasing in fluorescence following MHC1 staining. The shift in population signifies high levels of expression of MHC1 on the cell surface. Both 4T1 and CT26 cell lines demonstrated MHC1 expression and therefore hold the potential to interact with TCRs. The presence of MHC1 is vital for immunological studies, therefore its presence on both cell lines is vital to allow them to further be developed for tumour models. As expected, when stained for MHC2 expression, the results show no population shift, indicating no MHC2 expression on either 4T1 or CT26 cell lines. CT26 shows a large population shift when stained for CD80, depicting a high level of expression. No shift is seen for either CD86 or ICAM-1. However, the apparent presence of CD80 should be sufficient to induce CTL proliferation and effector function, deeming CD26 a potential novel tumour cell line model. In contrast, little/no convincing shifts are seen in 4T1 expression of co-stimulatory markers. Antibodies were titrated, to ensure the correct dilution was utilised. In addition, both accutase and trypsin were tested as means of cell detachment, to ensure that the lack of receptors was not due to cleavage via trypsin. The lack of co-stimulation molecules on 4T1 may present as an issue for its use in immune studies, therefore additional investigation must be undertaken, such as confluence dependent molecule expression.

The single stain flow cytometry results confirm the expression of PD-L1 on both 4T1 and CT26 cell lines, however no expression of Galectin-9. The expression of PD-L1 holds interesting prospects for future investigations into tumour:immune cell interactions, as well as potential investigations into the variation of PD-L1 expression due to different conditions and culture methods such as 3D spheroid cultures. Having PD-L1 expression further increases the interest in developing both 4T1 and CT26 cell lines into established tumour model for future use.

3.4.4 Are 4T1 and CT26 cells able to prime CL4 CD8+ T Cells?

Both 4T1 and CT26 cell lines have been reported to metastasis within murine models(180-181). The ability of metastasis enables migration of cancer cells into the tumour draining lymph nodes therefore giving the potential of direct presentation by the cancer cells to naive T cells. In order for cancer cells to prime naive T cells they must be able to provide 2 distinct stimulatory signals(87). The first signal is provided through the presentation of a recognisable antigen by a Class 1 MHC complex. All nucleated cells express major histocompatibility complexes; however, many cancers have been reported to down regulate MHC1 expression as an immune evasion mechanism(198).

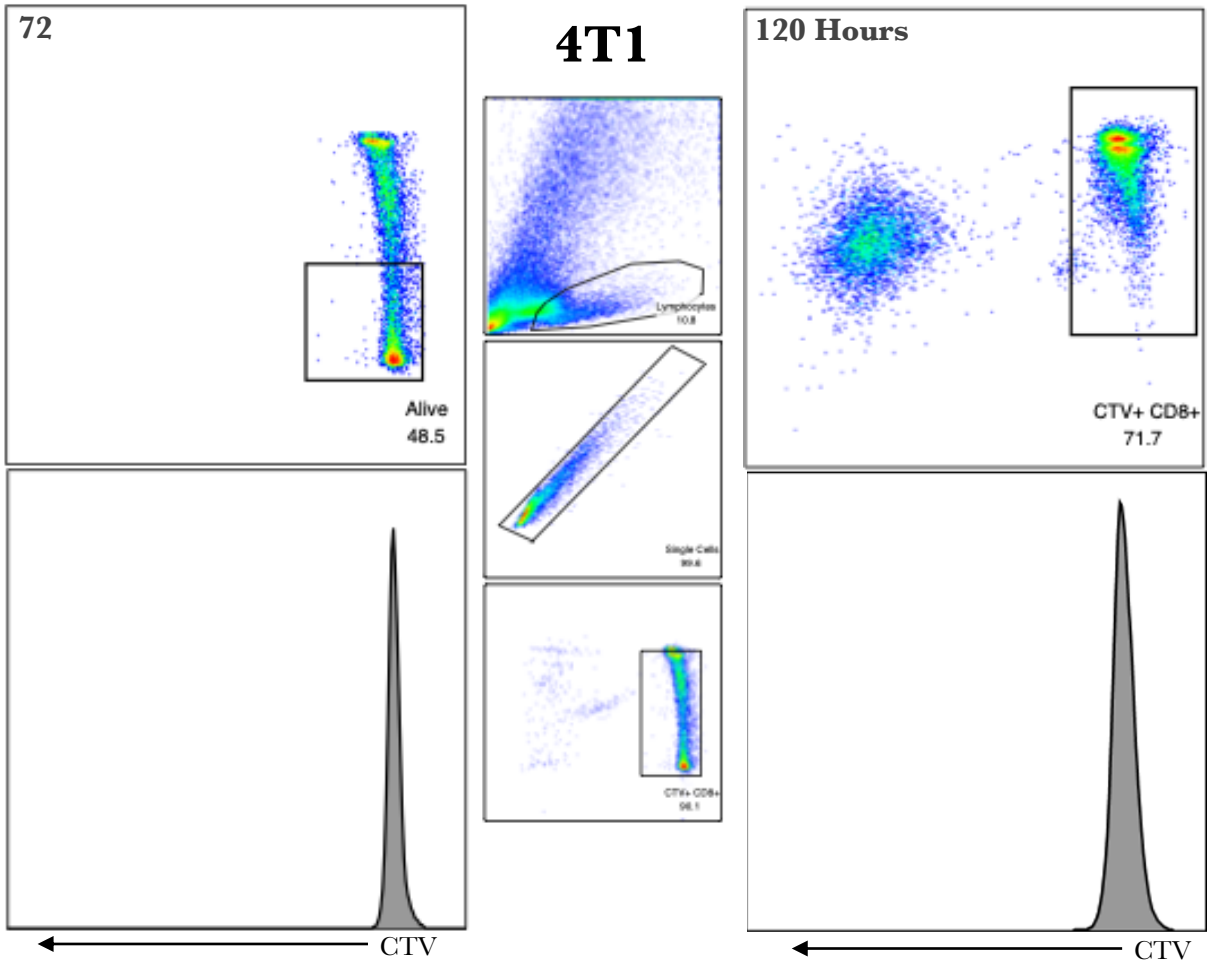
CT26 and 4T1 characterisation results show that both cell lines express high levels of MHC1 on their cell surface, enabling them to present the HA peptide following peptide loading. The second signal required for full activation of naive CTLs is provided via co-signalling receptors, most commonly CD80/CD86 on APCs, but also ICAM-1. A second stimulatory signal is essential for effective priming. CT26 cells were shown to express high levels of CD80. The expression of both MHC1 and CD80 by CT26 cells should be sufficient for the priming on naive CTLs once HA peptide loaded. In contrast, 4T1 cells were not shown to express CD80, CD86 or ICAM-1. The lack of expression of the named co-stimulatory receptors suggests that 4T1 may not be able to effectively prime naive T Cells. However additional co-stimulatory receptors, such as CD226, were not tested for therefore they still may be capable of inducing CTL proliferation(87). To determine whether CT26 and 4T1 cells are capable of priming naive T cells, flow cytometry proliferation assays were completed.

To demonstrate T cell proliferation naive CL4 splenocytes were stain using Cell Trace Violet(CTV) and co-cultured with irradiated 4T1 and CT26 cell lines. When cells undergo proliferation, they divide into two daughter cells including their cytoplasm. The division of a cell splits the level CTV stain into two producing a population of cells with lower levels of CTV and therefore lower levels of fluorescent emission. Decreases in emission enable the detection of whether a cell has undergone division, and therefore can be used to determine whether the cancer cells are

able to effectively prime naive T Cells. CT26 and 4T1 cell lines were irradiated to prevent their growth throughout the culture before being peptide loaded to present the HA antigen. Once loaded both the CTV stained naive CL4 splenocytes and the cell lines were plated in a 96 well plate at a 1:1 ratio. Assays were completed for both 72 and 120 hours to optimise the potential proliferation seen. Following incubation cultures were prepared with PI stain solution to enable detection of live/dead cells and run on the Novocyte flow cytometry system.

Results, depicted in figure 34, show that 4T1 cells are unable to induce CL4 CTL proliferation at both 72hrs and 120hrs. By 72 hours 48.5% of CL4 CTLs were still alive, however a single sustained CTV level demonstrated that no division had occurred. By 120 hours, all of the co-cultured CTLs were seen to be dead by the complete uptake of PI dye. Along with the total sample death, no proliferation had been witnessed over the extended culture period, thereby confirming that 4T1 cells were unable to induce CL4 CTL proliferation.

In contrast, by 72 hours CT26 92.6% of CL4 CTLs were alive, and were seen to have undergone one division event, indicating that CT26 were able to induce proliferation of CL4 CTLs. Following 120 hours of co-culture all of the CTLs had died, likely caused by the lack of nutrients in culture media as the larger CT26 cells require much higher levels of metabolites diminishing the supplies available for CTLs. No additional division events had occurred over the 120 hour period suggesting that lack of nutrients preceded any further potential proliferation.


72
Alive
48.5
4T1
120 Hours
CTV+ CD8+
71.7
CTV
CTV

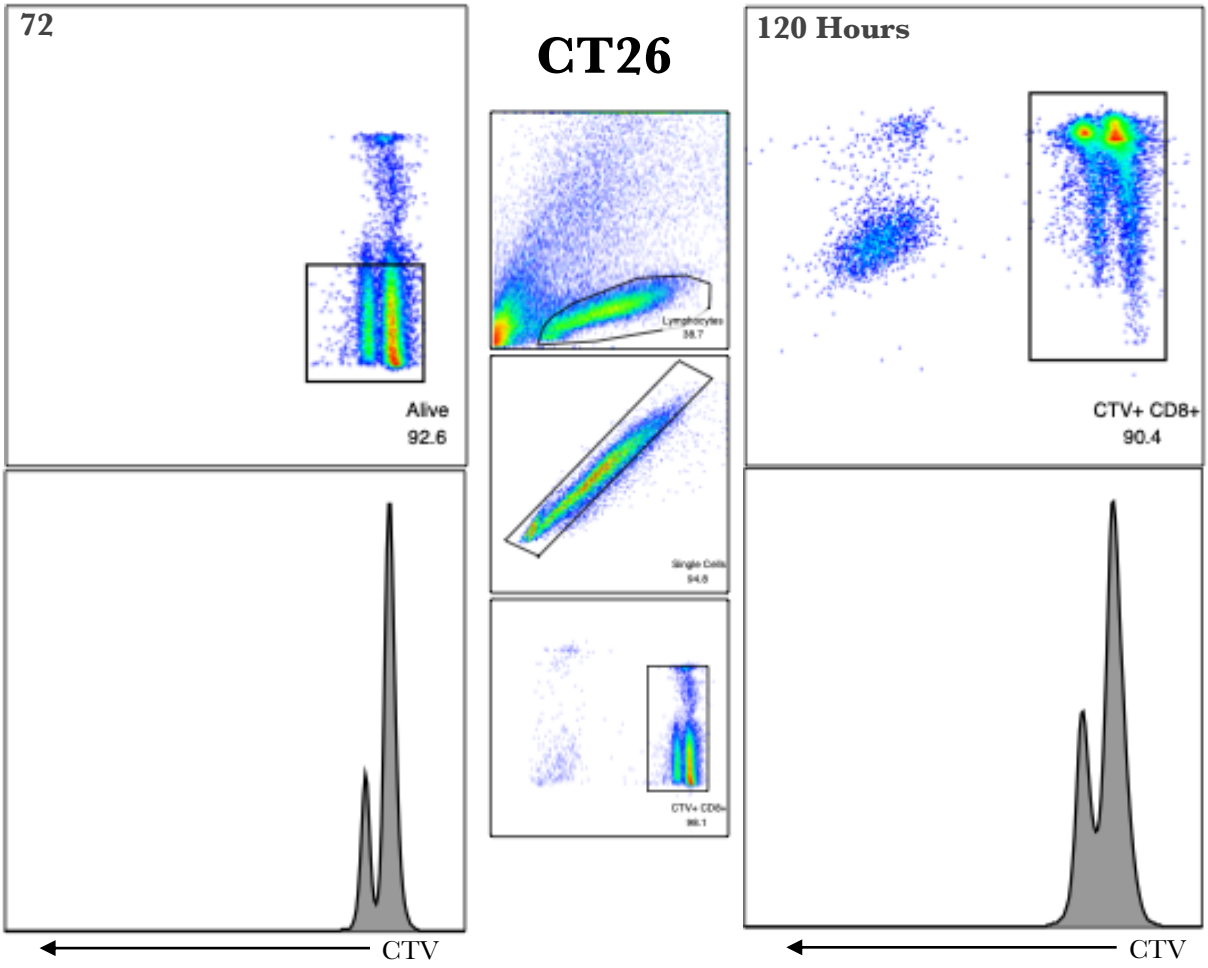

72
Alive
92.6
CT26
120 Hours
CTV+ CD8+
90.4
CTV
CTV

Figure 34: CTL priming via direct presentation from 4T1 and CT26 cancer cell lines

4T1 cells are not capable of inducing CD8+ T cell proliferation. 60-80% confluent 4T1 and CT26 cells were detached from T25 flasks using 500uL Trypsin-EDTA. Detached cells were quenched in 4mL complete media, collected into separate 15mL falcon tube and centrifuged. The cell lines were resuspended in 10mL complete media and transferred into 50mL falcon tubes. Cells were irradiated for 9680 seconds, 33cm away from the source, resulting in 9600 Rads. Once complete, cells were washed twice, resuspended in 1mL complete media and counted. Cells were further diluted with complete media to make $1x10^6$cells/mL. Naive CL4 splenocytes were harvested, counted and washed twice in PBS. Cells were centrifuged(1400rpm, 5min), resuspended in 600uL MACs buffer in addition to 50uL anti-CD8 MACs beads, and incubated for 30 minutes at $4^{o}C$. An LS column was placed in a midiMACs magnet, above an open 50mL falcon tube, and wetted using MACs buffer. Once incubation was complete, cells were washed twice in MACs buffer, and resuspended in 5ml. The solution was applied to the LS separation column. Once the sample had passed, the LS column was washed with 5mL MACs buffer. LS column was removed from the midiMACs magnet and 5mL MACs buffer added. A sterile plunger was forcefully pushed expelling solution through the column into a 50ml falcon tube, resulting in a CD8+ rich population of cells. Upon collection the CD8+ cells were washed using T cell media, counted and resuspended at $1x10^6$ cells/mL in T cell media. CTV stain was diluted 1uL in 1000uL PBS. 1uL CTV was used per $4x10^6$ cells. Splenocytes were centrifuged, supernatant discarded and resuspended in diluted CTV stain. CTV Staining was completed at $37^{o}C$ for 15 minutes, followed by a 5x quench in 0.5% protein rich media and an additional 15 minute, dark incubation at room temperature. When complete, cells were washed 3 times in RPMI, re-counted and resuspended at $5x10^5$ cells/mL in complete media. 100uL of either 4T1 or CT26 cells and CL4 CTLs were co-cultured together in individual wells of a 96 well plate and cultured for 72 or 120 hours. Following culture wells were vigorously resuspended, 200uL samples were extracted from each well and transferred into corresponding wells in a new 96 well plate. The new plate was centrifuged(1400rpm, 5 mins) and supernatant discarded. PI was diluted 1uL:400uL FACs buffer. Samples were washed twice in PBS and resuspended in 200ul PI solution, as described in methods. Samples were run on the Novocyte flow cytometer. Analysis utilised a gating strategy, shown in figure 19, to identify alive CL4 CTLs. Analysis of CTV emission determined 4T1 cells were not able to prime CL4 CTLs within 72 or 120 hours. In contrast, results show CT26 cells were able to prime the CTLs, inducing one proliferation event. In both cell line cultures, alive CL4 CTLs were present at 72 hours however by 120 hours all CL4 CTLs had died.(n=3)

3.4.5 Are CL4 CD8+ T cells able to eliminate 4T1 and CT26 cells?

In order to investigate the suppression of cytotoxicity seen in tumour infiltrating lymphocytes and therefore the suppressive mechanisms conjured by the tumour microenvironment, CTLs must first be able to be recognised and eliminate the cell lines.

To determine the cytotoxic effect of CL4 CTLs on both CT26 and 4T1 cells, kill assays were completed. The well-established Renca HA cells were used as a comparative control as previous experiments have demonstrated CTLs exert a consisted level of cytolysis when they are targeted. Target cells were stained with Cell Trace Violet to allow their detection and plated in a 96 well plate. CTLs were then counted and added onto the target cells at a 1:1 ratio. Co-cultures were incubated for 17 hours before being centrifuged and resuspended in accutase. Accutase detached the remaining adhered cells from the wells to enable PI staining. Resuspension in PI stain solution allowed immediate detection of live/dead cells when run on the Novocyte flow cytometer system, results shown in figure 35.

With the use of the well-established Renca HA cell line as a control, results shown clearly demonstrate CTLs are able to effectively eliminate both 4T1 and CT26. Comparable levels of specific cytotoxicity was noted when comparing 4T1(35.19%) and Renca cells(40.05%) ($p=0.8951$). However, a one way anova revealed a significant difference($p<0.0001$) between the groups. Further comparisons deemed the significant results due to the increased level of specific cytotoxicity of CT26 cells. The increase in cytotoxicity was seen to be significantly higher than seen in both 4T1($p<0.0001$) and Renca cells($p<0.0001$).

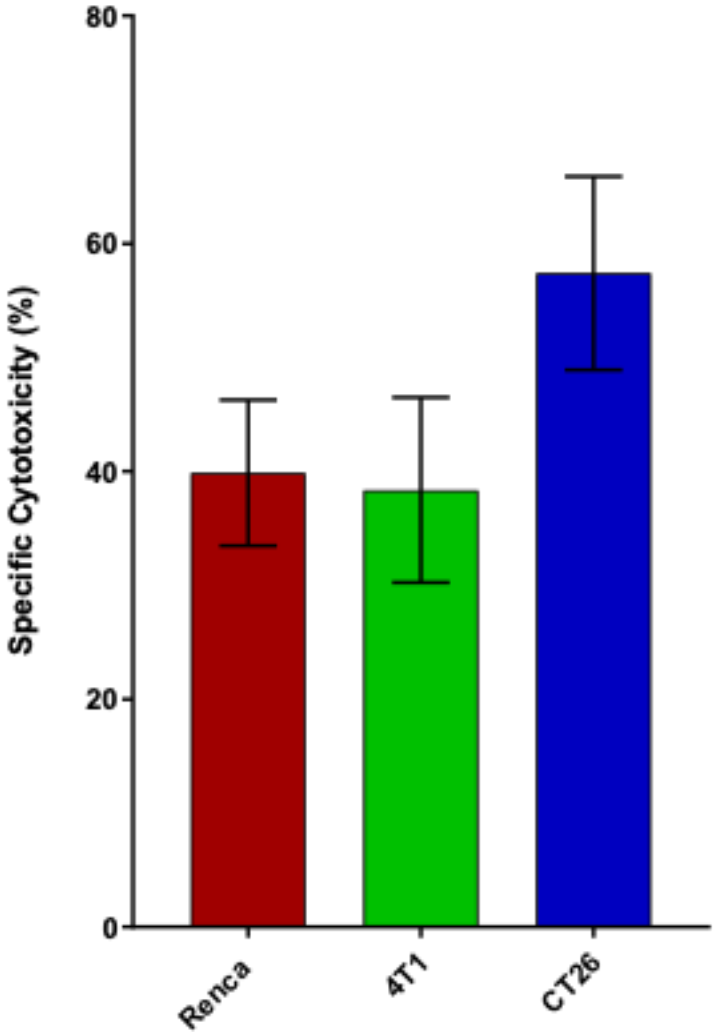


Figure 35: Comparative cell line cytolysis

Naive CL4 splenocytes were harvested and primed overnight using a mixed lymphocyte reaction with K^dHA, as described in methods. PGE_2 conditions(0.1uM PGE_2/1uM PGE_2/10uM PGE_2) were added into separate wells at time of priming. $5x10^6$ primed splenocytes per condition were harvested, washed and resuspended in 2ml IL-2 media with their corresponding conditions. The cellular suspensions were plated into separate wells of a 24 well plate and cultured for 96 hours. 60-80% confluent Renca HA cells were detached from a T25 flask using 1mL Trypsin-EDTA. Detached cells were quenched in 4mL complete media, collected into a 15mL falcon tube and centrifuged. Renca HA cells were resuspended in 1mL complete media and counted. Additional complete media was added until $1x10^6$ cells/mL was achieved. 2uL K^dHA was added to the suspension, and incubated for 60 minutes, at 37^{oC}. Once complete cells were centrifuged(1200rpm, 3min), supernatant discarded and the cells washed twice in PBS. CTV stain was diluted 1uL in 1000uL PBS. 1uL CTV was used per $1x10^6$ cancer cells. Renca HA cells were counted, centrifuged, supernatant discarded and resuspended in diluted CTV stain. CTV Staining was completed at 37^{oC} for 15 minutes, followed by a 5x quench in 0.5% protein rich media and an additional 15 minute, dark incubation at room temperature. When complete, cells were washed twice in complete media, re-counted and resuspended to $1x10^6$ cells/mL in complete media. All cultured CTLs were harvested into separate 15mL falcon tubes corresponding to their culture conditions. All CTLs were washed in RPMI, and counted. CTLs were further washed and resuspended to $1x10^6$ cells/mL in complete media. 100ul of both cancer cells and CTLs were co-cultured in repeated wells of a 96 well plate. 4 repeats were plated per condition, in addition to 4 wells of Renca HA only and 4 wells for a positive killed control.

The plate was incubated at 37^{oC} for 17 hours. When complete, media from the positive control wells was carefully extracted ensuring not to disturb the adherent cells. 150ul accutase was added to the positive control wells and incubated for 10 minutes at room temperature. Positive control wells were resuspended and collected into a 1.5mL eppendorf. The eppendorf was heated to 90^{oC} for 10 minutes in a heat plate. The remaining plate was centrifuged at 1400rpm 5 mins, and supernatant discarded. PI was diluted 1uL:400uL in accutase. 150uL PI solution was added to each remaining well, incubated for 10 minutes at room temperature and run within the hour. PI was added to the heated cell accutase at a 1uL:400uL ratio. 150uL kill positive cells were pipetted into a new row of wells in the 96 well plate. All wells were resuspended and run on the Novocyte. Gating strategy seen in figure 19 was used to identify target cells including their live/dead populations. Percentage of dead target cells was determined and specific cytotoxicity calculated using the formula, noted in methods. A one-way Anova demonstrated a significant difference($n=3$, $p<0.0001$) of cytolysis between the cell lines. Individual comparisons show no significant difference between the percentage of CL4 CTL specific cytotoxicity against 4T1 and Renca cell lines($p=0.8951$). CT26 showed a substantial increase in CTL specific cytolysis compared to both Renca($p<0.0001$) and 4T1($p<0.0001$).

3.4.6 Discussion

Cancer is described as a collection of over 200 individual diseases characterised by their aberrant growth and accelerated proliferation. All cancers share a collection of phenotypic characteristics, deemed the "hallmarks of cancer"(2). One more recently discovered hall mark is the escape and evasion of the immune system(23). However, there are extensive mechanisms behind which cancer cells evade the immune system, many of which vary depending on the type of cancer. Many up regulate the expression of immunosuppressive ligands such as PD-L1, whereas others increase intracellular proteins, e.g. COX-2, thereby increasing the local concentration of PGE2(200, 186). Therefore, in order to complete translational research multiple tumour types must be investigated to determine whether any effects seen are global or specific to a single cancer type.

The study completed, developed and investigated the immunological properties of two new cancer cell lines in order for their future use. 4T1 and CT26 cancer cells were extensively cultured and properties investigated to determine whether they would be useful in future immunological studies. 4T1 cells are of epithelial origin from a murine mammary carcinoma. Whereas the CT26 cell line is fibroblastic from a colorectal carcinoma. Both cell lines were very simple to culture,

however, grew at very different rates. 4T1 cells grew extremely quickly yet experienced cell death if 100% confluence was exceeded. In contrast, CT26 cells did not grow as quickly yet once 100% confluence was reached, cells would grow onto of each other creating dense fibroblastic mesh. Both cells have been reported to metastasis extensively, and therefore have great potential for in vivo murine studies.

Characterisation of the cell lines provided basic descriptions of key cell surface molecules on the differing cell lines. As both expressing MHC1, they are capable of presenting intracellular peptides to CTLs, giving them potential in immunological studies. However, for complete interaction with CTLs co-signalling molecules must also be apparent. CT26 express high levels of CD80, making it a very good model for interactions with CD8+ T cells. Proliferation assays further supported CT26 as a good candidate for immune studies as CTL proliferation was seen upon co-culture of HA pulsed CT26 cells with naive T cells. In contrast 4T1 cells did not notably express any tested co-stimulatory molecules. There are multiple additional co-signalling molecules that were not tested for therefore further investigations must be completed before disregarding the 4T1 cell line as a model for immunological studies. However, no proliferation was seen when HA pulsed 4T1 cells were co-cultured with naive T cells, deeming the model in comparable with the current Renca model when studying immune reactions. In spite of its comparability, 4T1 cells have been reported to extensively metastasis in murine models, providing the potential for alternative immune studies to be completed. Modern techniques of inducing foreign protein expression into cells, such as lipofection and CrispR-Cas9, have great potential to induce co-stimulatory signal expression in 4T1 cells, providing an increasingly sophisticated model. The introduction of individual co-stimulatory receptor expression would allow the investigation and comparison of immune interactions with different co-signalling receptors and their effectiveness/suppression, thereby enabling further controlled research models.

Kill assays demonstrated comparable results of cancer cell elimination to the currently used Renca model. Pulsed 4T1 cells were eliminated at indistinguishable levels to Renca HA cells,

whereas the CT26 cell line exhibited higher levels of cytolysis. The high level of cytolysis is likely due to the increased level of CD80 expression. CD80 is able to efficiently activate CTLs enabling their effective cytolytic action and elimination of cancer cells. In addition, it was be noted that 4T1 cells may express a higher level of PD-L1. Increased PD-L1 expression can induce increased levels of CTL cell death upon interaction, therefore decreasing the level of cytolysis witnessed.

In conclusion both cell lines have great promise for use in immunological studies. The CT26 line provides an interesting comparison to the currently used Renca model, due to its expression of classical co-stimulatory receptor CD80 compared to Renca's ICAM-1 signalling mechanism. In addition, its fibroblastic cell morphology enables further research into the contributing effects of different cell types in tumour induced immunosuppression. In contrast 4T1 cells provide an interesting model of in vivo metastasis, and immune evasion. Due to their relatively low expression of classical co-signalling molecules, the introduction of alternative co-signalling molecules would enable extremely controlled immune experiments into the contribution of alternative co-signalling molecules in immune stimulation and suppression. However, to utilise the full potential of both cell lines with the CL4 model, they must be transfected to express the KdHA peptide to enable CL4 in vivo studies, and abolish the need for peptide loading. Furthermore, the producing of mCherry emitting 4T1 and CT26 cell lines would enable their use in the optimised IncuCyte imaging based kill assay, giving further understanding into their elimination by CL4 CTLs.

4. Future work

4.1 Investigating PGE2 induced immunosuppression

Overall results from the project confirm that PGE2 is capable of suppressing both the proliferation and the cytolytic effector function of cytotoxic T cells. However, one key limitation noted throughout the project was the circumvention of suppression seen when high levels of IL-2 were used to supplement the culture media. CTLs cultured in IL-2 showed little/no effect when cultured in levels of PGE2(1uM) that had previously been reported to induce significant suppression(91). 10-fold higher levels of PGE2 were needed to witness significant suppression that matches the literature. The results of the project would imply that the addition of IL-2 in CTL culture has inhibited suppression that may be noted in more physiological systems, resulting in false negative results. In addition, IL-2 cultured CTLs have been used as a comparative control with exceedingly high cytolytic function, thereby potentially witnessing false positive results. The new developed method of culturing T cells acts as a more physiological control; however, this must be confirmed. Future research must be completed into determining and optimising the most physiologically relevant culture method. Kill assay comparisons between mature effector/effector memory CL4 CTLs and the ex vivo cultured CTLs should be examined to decipher the most accurate method of culture. In addition, the previous Wuelfing CTL culture method should be characterised over a time course to determine the precise differentiation of the population of CTLs being produced. Furthermore, the newly developed culture method is needed to be fully optimised, to enable a high yield of effector/effector memory cells coupled with an increased survival rate, for utilisation in kill assays. Once fully optimised, more physiologically primed CTLs should also be used in imaging studies to fully assess T cell signalling, polarisation and the suppressed phenotype seen.

Additional studies into the suppression induced by PGE2 should also be completed. The noted addition of TG4-155, partially resolving suppression can be explained by the interaction of PGE2 with alternative EP receptors, namely EP4, however cannot be confirmed. Further experiments

should be completed, utilising secondary EP blockers, as well as the identification of specific EP receptor expression on the CTL cell surface. Understanding the EP receptor dynamics would allow for a more in-depth understanding on the suppressive effects exerted by PGE2 within the tumour microenvironment.

In vivo studies of PGE2 would not only circumvent the immediate need for a physiologically relevant CTL culture method but would also allow investigations into the role of PGE2 in the tumour microenvironment as a whole. Development and inoculation of a murine model with a COX-2 k/o cancer cell line would allow detection of the contribution of PGE2 in the induction of the suppressed phenotype seen in tumour infiltrating lymphocytes.

4.2 Development of new cancer cell lines

The newly developed cancer cell lines show great promise for future immunological studies. In order to fully maximise their potential transfection of the KdHA protein should be completed to allow for both: in vivo investigations, as well in vitro CL4 studies without the need for loading. Cytoplasmic staining of cells for use in Incucyte kill assays has been attempted however the process of staining using chemicals such as CFSE proves to affect the cells, making them less adherent and not in optimal state, therefore adding an additional unreliable variable to the results. Therefore, once the cells are eble to endogenously present the HA peptide, an additional transfection for mCherry cytoplasmic fluorescence would be extremely useful as it would enable their use in IncuCyte imaging-based kill assays.

5. Conclusion

This study aimed to determine the effect of PGE_2 on CTL function as a possible mechanism of tumour mediated immune-suppression. Overall the study confirms that PGE_2 does induce

significant suppression of CTL proliferation and cytotoxic function, and therefore could play a role in inducing suppressed TIL function. However, the effect of PGE_2 was shown to be entirely dependant on the culture conditions of CTLs. Increased IL-2 supplementation of CTL culture abolished any noted suppression, requiring a 10-fold increase in PGE_2 concentration before suppression was seen. Utilising a new culture method allowed identification of significant suppression by 1uM PGE2, supporting current literature, whilst maintaining effective levels of proliferation and cytolysis in untreated control. PGE_2 was did not demonstrate any effect on the immunological synapse, suggesting an alternative mode of action, however this may be down to culture method and should therefore be retested utilising the new culture method. Two new cell lines , 4T1 and CT26, show great promise in future comparative immunological studies, due to their contrasting co-signalling molecule expression, yet maintained levels of elimination via CTLs.

6. Appendix

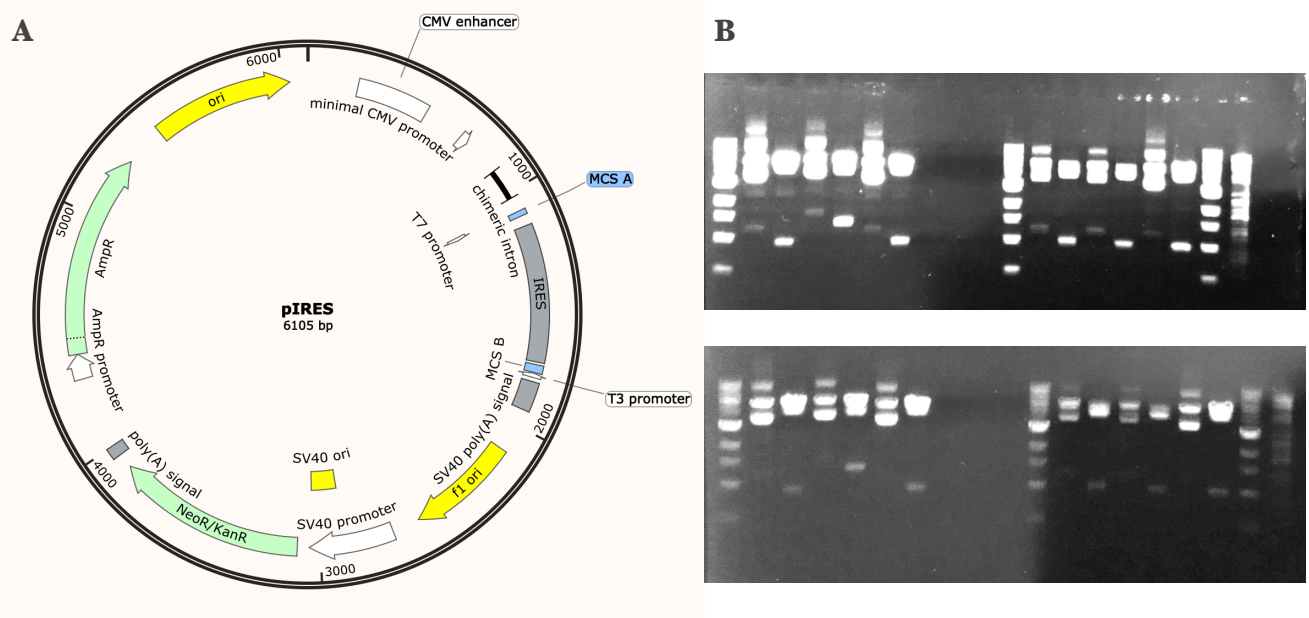


Figure: Transfection of 4T1 and CT26 cell lines

The CL4 mouse model enables immunological studies through the use of a monoclonal population of CD8+ T cells, uniformly expressing a TCR specific for the haemagglutinin(HA) antigen from the influenza virus(A/PR/8/H1N1). Utilising a monoclonal population of CTLs allows for the investigation of specific T cell interactions with any cells expressing the HA antigen and co-signaling receptors, including cancer cells. To optimise the experimental use of 4T1 and CT26 cell lines in the CL4 model, including murine inoculation and tumour formation, they must endogenously present the HA antigen, allowing for recognition and interaction by CL4 CD8+ T cells. The currently used Renca cell line was previously transfected to express the HA peptide, as well a multiple other characteristic such as the over expression of ICAM-1. Previous transfection was completed via plasmid lipofection. A frozen glycerol stock of the, previously used, HA coding p-IRES vector was located in the Morgan lab -80c freezer.

In attempt to isolate the vector, the glycerol stock was thawed and plated on Ampicillin supplemented agar and allowed to grow. Solitary colonies were isolated, ensuring origination from a single cell, used to inoculate bacteria growth media and left overnight in a shaking incubator to permit maximal growth. Following culture, the bacterial cells were re-isolated. A centrifuge mini prep(Qiagen) was completed to extract and elute the vector. Once isolated, the vector was both digested and linearized before being run through gel electrophoresis to confirm the correct vector was present. Restriction Enzymes NDE1 and STU were use in attempt to cut out the multiple cloning sites, as seen in figure 24, and therefore potential location of the HA peptide. Empty p-IRES vectors, also from frozen glycerol stocks, were used as controls.

3ul NDE1 and STU were added to 3ul of eluted plasmid DNA and incubated for 1hr at 37c. A control of 3ul of the undigested of DNA was diluted in ddH20. Agarose powder and ddH2O was mixed and solution gently warmed until fully dissolved. Clear solution was poured into a gel template and wells created using a pronged comb. Once solid, the gel was placed onto an electrophoresis unit and submerged in 1% TAE. All samples were mixed with loading dye and pipetted into their corresponding wells, at the negative end of unit. 1Kb molecular weight ladders were loaded either sides of the samples to enable the calculation of their sizes. Electrical current was tippled and gel run for 1 hour. Upon completion the gel was removed from the unit and photographed using a Transilluminator for imaging and analysis. Results show an aberrant number and size of DNA segments, determining the plasmid not to be pIRES-HA.

Analysis of the gel electrophoresis results indicate no presence of the HA peptide within the vector. Furthermore, upon further investigation, the results indicate that the DNA seen was not the expected vector. Both the control empty p-IRES and p-IRES-HA showed no associated of banding following digestion or linearisation. 1Kb ladders were used to calculate the length of DNA, however both the size and number of noted fragments did not correlate to the expected p-IRES vectors. Measures were taken to ensure this was not due to contamination. Further investigations lead to the completion of external DNA sequencing, which confirmed that the DNA acquired was not the desired plasmid, sequence shown in appendix. Novel HA expressing vectors have since been designed and purchased to ensure lipofection, if successful, resulting in expression of the correct HA peptide, enabling endogenous recognition via CL4 CTLs. Until the new vector arrives and is lipofected, investigations shall rely on peptide pulsing to induce CTL responses, which has proven successful.

NNCGAGCTCGGNTCGANATCTGCGGCCTAGCTAGCGCTTAAGGCCTGTTAACCGGTCGTACGTCT
CCGGATTCGAATTCGGATCCGCGGCCGCATAGATAACTGATCCAGTGTGCTGGAATTAATTCGCT
GTCTGCGAGGGCCAGCTGTTGGGGTGAGTACTCCCTCTCAAAAGCGGGCATGACTTCTGCGCTAA
GATTGTCAGTTTCCAAAAACGAGGAGGATTTGATATTCACCTGGCCCGCGGTGATGCCTTTGAGG
GTGGCCGCGTCCATCTGGTCAGAAAAGACAATCTTTTTGTTGTCAAGCTTGAGGTGTGGCAGGCT
TGAGATCTGGCCATACACTTGAGTGACAATGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCA
CTCCCAGGTCCAACTGCAGGTCGAGCATGCATCTAGGGCGGCCAATTCCGCCCCTCTCCCCCCNN
CCCCTCTCCCTCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGT
TTGTCTATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGC
CCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTT
GAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGACCC
TTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAA
GATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGT
CAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGANNGTACNCCATTGTA
TGGGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTC
TAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAANNNGATGA

NTNNATNNCTGCGGCCTAGCTAGCGCTTAAGGCCTGTTAACCGGTCGTACGTCTCCGGATTCGAA
TTCAACAAATTAGAAAAAAGGATGGAAAATTTAAATAAAAAAGTTGATGATGGATTTCTGGACAT
TTGGACATATAATGCAGAATTGTTAGTTCTACTGGAAAATGAAAGGACTCTGGATTTCCATGACT
CAAATGTGAAGAATCTGTATGAGAAAGTAAAAAGCCAATTAAAGAATAATGCCAAAGAAATCGGA
AATGGATGTTTTGAGTTCTACCACAAGTGTGACAATGAATGCATGGAAAGTGTAAGAAATGGGAC
TTATGATTATCCCAAATATTCAGAAGAGTCAAAGTTGAACAGGGAAAAGGTAGATGGAGTGAAAT
TGGAATCAATGGGGATCTATCAGATTCTGGCGATCTACTCAACTGTCGCCAGTTCACTGGTGCTT
TTGGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGTTCTAATGGATCTTTGCAGTGCAGAATATG
CATCTGAGGATCCGCGGCCGCATAGATAACTGATCCAGTGTGCTGGAATTAATTCGCTGTCTGCG
AGGGCCAGCTGTTGGGGTGAGTACTCCCTCTCAAAAGCGGGCATGACTTCTGCGCTAAGATTGTC
AGTTTCCAAAAACGAGGAGGATTTGATATTCACCTGGCCCGCGGTGATGCCTTTGAGGGTGGCCG
CGTCCATCTGGTCAGAAAAGACAATCTTTTTGTTGTCAAGCTTGAGGTGTGGCAGGCTTGAGATC
TGGCCATACACTTGAGTGACAATGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAG
GTCCAACTGCAGGTCGAGCATGCATCTAGGGCGGCCANTNCGCCN

Figure 37 Plasmid Sequencing:

Midi-prep isolated plasmids were sent for external DNA sequencing. Sequencing was completed by Eurofins genomics, and results electronically returned. Results demonstrated no presence of the HA peptide within the plasmids. In addition no IRES component was noted, deeming the plasmid incorrectly identified.

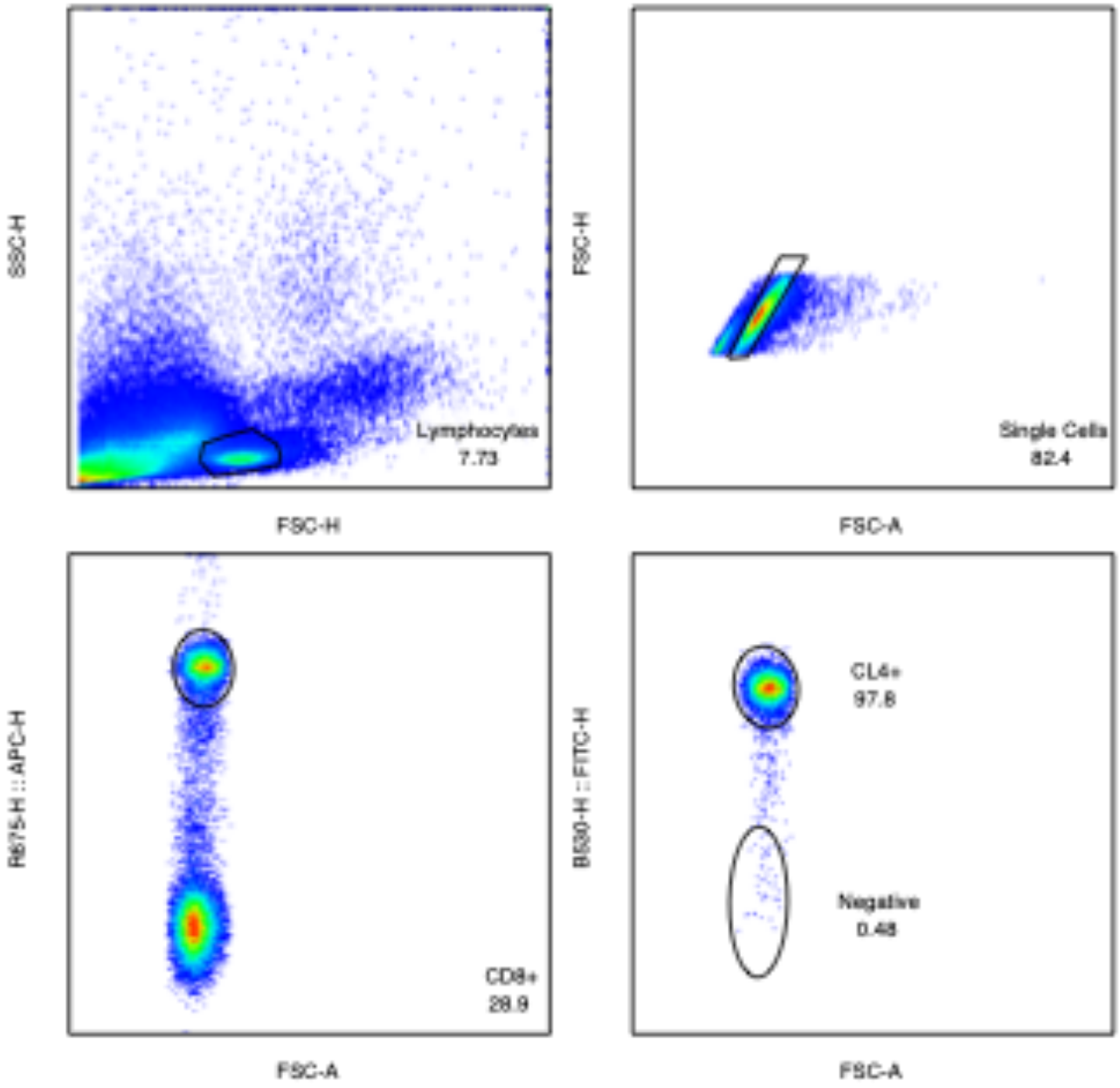
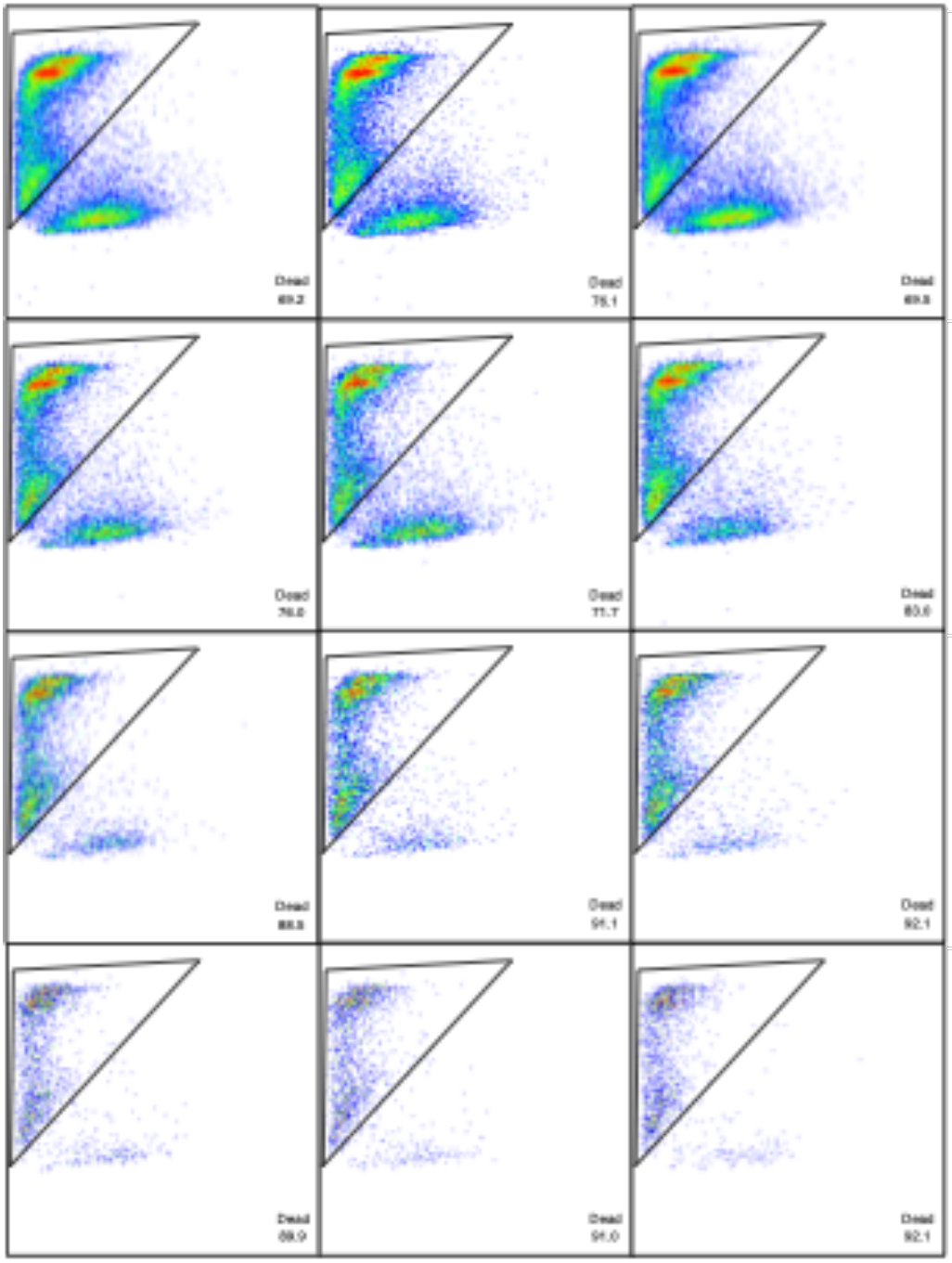


Figure 36: Genotyping CL4+ mice.

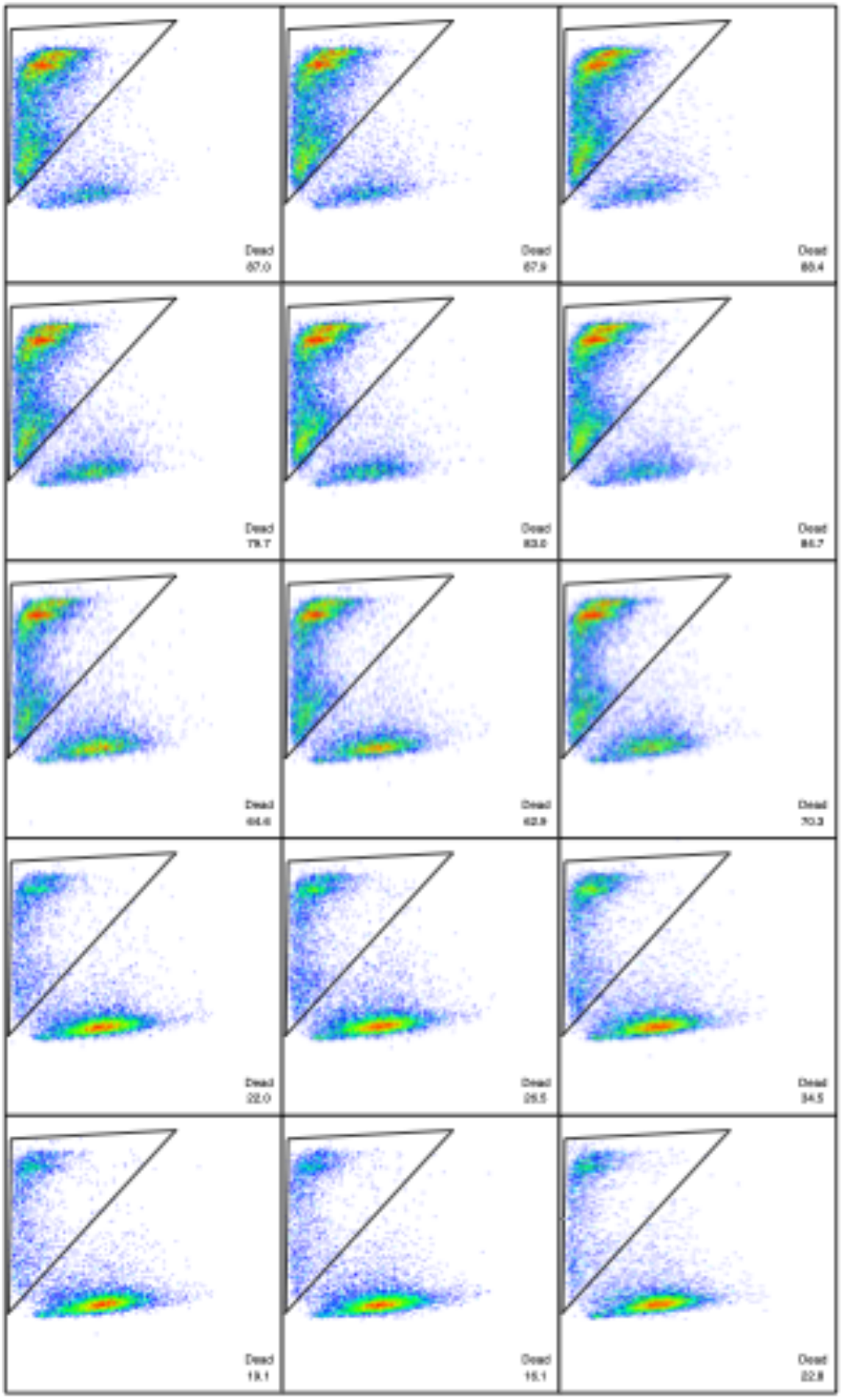
Blood extracted through the tail vein of each mouse was diluted using 2mL FACS buffer. Samples were centrifuged(1400rpm, 5min) and supernatant discarded. Cells were resuspended in 1mL Ack Lysis buffer for 3 minutes. Cells were washed twice using FACS buffer. 1uL CD8+ stain(APC) and Vβ8.1 stain(FITC) were diluted 1uL in 100uL FACs buffer. Samples were centrifuged, supernatant discarded, and resuspended in 50uL diluted stain for 30 minutes at 4^{oC}. Samples were washed twice and resuspended in 200uL FACs buffer. Lymphocytes were determined based on cell size and granularity(FSC-H/SSC-H). Single cells were detected from the lymphocyte population using FSC-H/FSC-A. CD8+ cells were isolated, by an increase in APC fluorescence. CD8+ cells were analysed for their Vβ8.1 expression, indicated by increased FITC fluorescence emission. Genotyping identified $CL4^{+/-}$ mice by over 90% of the CD8+ population being Vβ8.1+.

A



CL4 CTL	Target
1	2
1	1
1	0.5
1	0.125

B



CL4 CTL	Target
5	1
1	1
0.5	1
0.25	1
0.125	1

Figure 35: Killing assay - Target cell titration.

Naive CL4 splenocytes were harvested and primed overnight using a mixed lymphocyte reaction with K^dHA, as described in methods. The cellular suspensions were plated into separate wells of a 24 well plate and cultured for 96 hours. 60-80% confluent Renca HA cells were detached from a T25 flask using 1mL Trypsin-EDTA. Detached cells were quenched in 4mL complete media, collected into a 15mL falcon tube and centrifuged. Renca HA cells were resuspended in 1mL complete media and counted. Additional complete media was added until $1x10^6$ cells/mL was achieved. 2uL K^dHA was added to the suspension, and incubated for 60 minutes, at 37^{oC}. Once complete cells were centrifuged(1200rpm, 3min), supernatant discarded and the cells washed twice in PBS. CTV stain was diluted 1uL in 1000uL PBS. 1uL CTV was used per $1x10^6$ cancer cells. Renca HA cells were counted, centrifuged, supernatant discarded and resuspended in diluted CTV stain. CTV Staining was completed at 37^{oC} for 15 minutes, followed by a 5x quench in 0.5% protein rich media and an additional 15 minute, dark incubation at room temperature. When complete, cells were washed twice in complete media, re-counted and resuspended to $1x10^6$ cells/mL in complete media. All cultured CTLs were harvested into a 15mL falcon tube, washed in RPMI, and counted. CTLs were further washed and resuspended to $1x10^6$ cells/mL in complete media. Different ratios of cancer cell:CTL were co were co-cultured in repeated wells of a 96 well plate. 3 repeats were plated per condition, in addition to 3 wells of Renca HA only and 3 wells for a positive killed control. The plate was incubated at 37^{oC} for 17 hours. When complete, media from the positive control wells was carefully extracted ensuring not to disturb the adherent cells. 150ul accutase was added to the positive control wells and incubated for 10 minutes at room temperature. Positive control wells were resuspended and collected into a 1.5mL eppendorf. The eppendorf was heated to 90^{oC} for 10 minutes in a heat plate. The remaining plate was centrifuged at 1400rpm 5 mins, and supernatant discarded. PI was diluted 1uL:400uL in accutase. 150uL PI solution was added to each remaining well, incubated for 10 minutes at room temperature and run within the hour. PI was added to the heated cell accutase at a 1uL:400uL ratio. 150uL kill positive cells were pipetted into a new row of wells in the 96 well plate. All wells were resuspended and run on the Novocyte. Gating strategy seen in figure 16 was used to identify target cells including their live/dead populations. Percentage of dead target cells was determined and specific cytotoxicity calculated using the formula, noted in methods.(n=2)

A. Target cell titrations determined that a 1:1 CTL to Target cell ratios gave the cleanest separation whilst maintaining a sizeable alive target cell population enabling easy detection of both live and dead populations and therefore specific cytotoxicity calculations.

B. Killer cell titrations determined 1:1 CTL to target cell ratio to be the most efficient. A 5:1 ratio showed slightly increased, but not significant, levels of killing but required notably higher numbers of CTLs. A 1:1 ratio maintained high levels of killing without the need for excessive CTLs and was therefore used throughout the project.

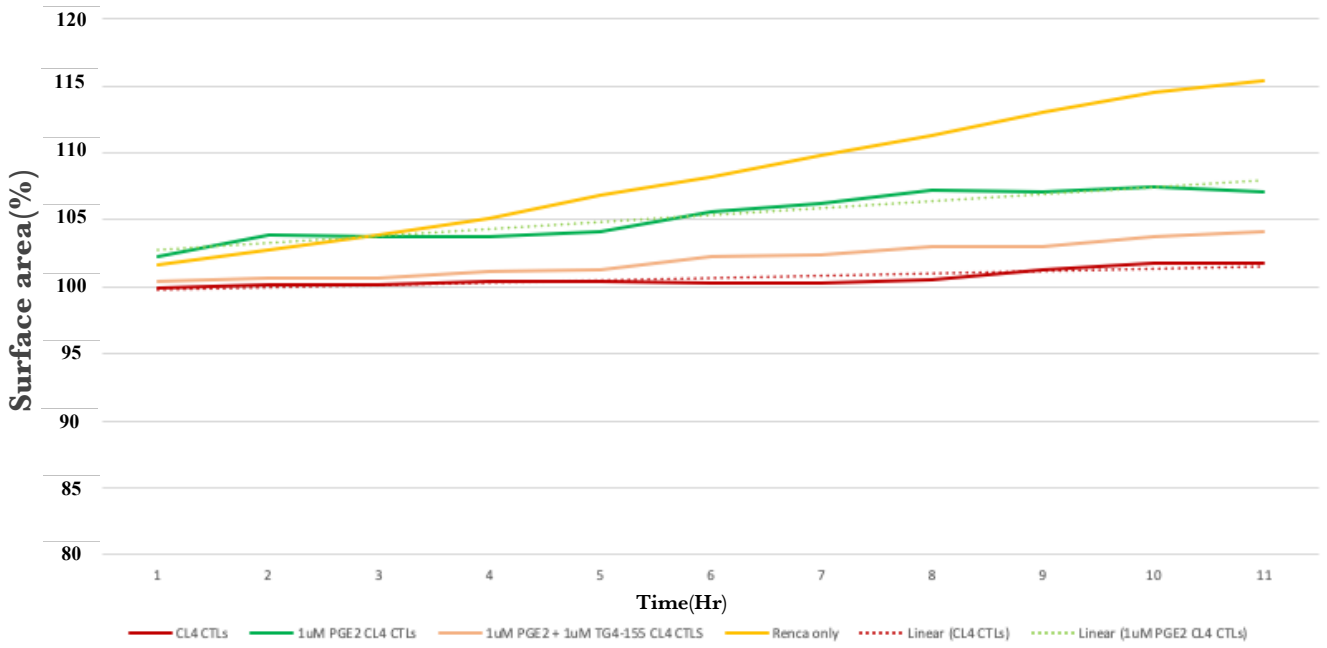


Figure 38: Killing assay of CL4 cultured using he previous culture method in absence of IL-2.

Naive CL4 splenocytes were harvested and primed overnight using a mixed lymphocyte reaction with K^dHA, as described in methods. PGE_2 conditions(0uM PGE_2/1uM PGE_2) were added into separate wells at time of priming. $5x10^6$ primed splenocytes per condition were harvested, washed and resuspended in 2ml T cell media with their corresponding conditions. The cellular suspensions were plated into separate wells of a 24 well plate and cultured for 48 hours. 60-80% confluent Renca mCherry cells were detached from a T25 flask using 500uL Trypsin-EDTA. Detached cells were quenched in 4mL complete media, collected into a 15mL falcon tube and centrifuged. Renca mCherry cells were resuspended in 1mL complete media and counted. Additional complete media was added until $1x10^6$ cells/mL was achieved. 2uL K^dHA was added to the suspension, and incubated for 60 minutes, at 37^{oC}. Cells were centrifuged, supernatant discarded and washed twice using 10mL RPMI. Once washed, Renca mCherry cells were re-counted, and resuspended to $3x10^5$ cells/mL in Fluorobrite media. 50uL Renca mCherry cells were plated in individual wells of a glass bottomed 384 well plate, and incubated until cellular adherence, at 37^{oC}. 4 repeats per condition were plated. All cultured CTLs were harvested into separate 15mL falcon tubes corresponding to their culture conditions. All CTLs were washed in PBS, and resuspended in 1mL conditioned imaging buffer. 1.5mL eppendorf were prepared containing 200uL 2X conditioned imaging buffer, for each experimental condition.CTLs were sorted using FACs for 60,000 alive cells per condition into their corresponding eppendorf, taking into account an un-pulsed control group. Sorted CTLs were centrifuged, 4000xg 4minutes, using a microfuge, and supernatant carefully extracted ensuring no disturbance to the cellular pellet. All CTLs were resuspended to $3x10^5$ cells/mL in 2X conditioned Fluorobrite media. 50uL CTLs were added to corresponding target wells containing adhered Renca mCherry, immediately before the plate was placed in the IncuCyte imaging system for 18 hours. Total target cell surface area(um^2/well) was measuring using red fluorescence masking, over the duration of the assay. The mean of 4 repeats was calculated per timepoint for each condition. Results were normalised, and rolling averages produce.

A Renca only control(Yellow) was used to demonstrate cell growth over the duration of the assay. The decrease in target cell surface area was indicative of target cell death via CTL cytolysis. CTLs treated with 1uM PGE_2(Green) saw a reduction in cytotoxicity compared to untreated CL4(Red). The addition of 1uM TG4-155 to 1uM PGE_2 treated samples(Orange) saw a partial reversal of the suppression seen, increasing cytolysis to comparable levels as the untreated control.(n=1)

7. References

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